



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification⁴ : C12Q 1/68, C07H 21/00</p>	A1	<p>(11) International Publication Number: WO 88/ 03957</p> <p>(43) International Publication Date: 2 June 1988 (02.06.88)</p>																								
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top; border-right: 1px solid black; padding: 5px;"> <p>(21) International Application Number: PCT/US87/03009</p> <p>(22) International Filing Date: 24 November 1987 (24.11.87)</p> <p>(31) Priority Application Numbers: 934,244 083,542</p> <p>(32) Priority Dates: 24 November 1986 (24.11.86) 7 August 1987 (07.08.87)</p> <p>(33) Priority Country: US</p> <p>(60) Parent Application or Grant (63) Related by Continuation US 083,542 (CIP) Filed on 7 August 1987 (07.08.87)</p> <p>(71) Applicant (for all designated States except US): GEN-PROBE INCORPORATED [US/US]; 9880 Campus Point Drive, San Diego, CA 92121 (US).</p> </td> <td style="width: 50%; vertical-align: top; padding: 5px;"> <p>(72) Inventors; and (75) Inventors/Applicants (for US only) : HOGAN, James, John [US/US]; 2727 First Avenue, San Diego, CA 92103 (US). SMITH, Richard, Dana [US/US]; 3522 Mercer Lane, San Diego, CA (US). KOP, Jo, Ann [US/US]; 1314 Hampton Road, San Marcos, CA 92069 (US). McDONOUGH, Sherrol, Hoffa [US/US]; 4697 Robbins Street, San Diego, CA 92122 (US).</p> <p>(74) Agents: KENNEY, J., Ernest; 625 Slaters Lane, Fourth Floor, Alexandria, VA 22314 (US) et al.</p> <p>(81) Designated States: AU, DK, FI, JP, KR, NO, US.</p> <p>Published <i>With international search report.</i></p> </td> </tr> </table>			<p>(21) International Application Number: PCT/US87/03009</p> <p>(22) International Filing Date: 24 November 1987 (24.11.87)</p> <p>(31) Priority Application Numbers: 934,244 083,542</p> <p>(32) Priority Dates: 24 November 1986 (24.11.86) 7 August 1987 (07.08.87)</p> <p>(33) Priority Country: US</p> <p>(60) Parent Application or Grant (63) Related by Continuation US 083,542 (CIP) Filed on 7 August 1987 (07.08.87)</p> <p>(71) Applicant (for all designated States except US): GEN-PROBE INCORPORATED [US/US]; 9880 Campus Point Drive, San Diego, CA 92121 (US).</p>	<p>(72) Inventors; and (75) Inventors/Applicants (for US only) : HOGAN, James, John [US/US]; 2727 First Avenue, San Diego, CA 92103 (US). SMITH, Richard, Dana [US/US]; 3522 Mercer Lane, San Diego, CA (US). KOP, Jo, Ann [US/US]; 1314 Hampton Road, San Marcos, CA 92069 (US). McDONOUGH, Sherrol, Hoffa [US/US]; 4697 Robbins Street, San Diego, CA 92122 (US).</p> <p>(74) Agents: KENNEY, J., Ernest; 625 Slaters Lane, Fourth Floor, Alexandria, VA 22314 (US) et al.</p> <p>(81) Designated States: AU, DK, FI, JP, KR, NO, US.</p> <p>Published <i>With international search report.</i></p>																						
<p>(21) International Application Number: PCT/US87/03009</p> <p>(22) International Filing Date: 24 November 1987 (24.11.87)</p> <p>(31) Priority Application Numbers: 934,244 083,542</p> <p>(32) Priority Dates: 24 November 1986 (24.11.86) 7 August 1987 (07.08.87)</p> <p>(33) Priority Country: US</p> <p>(60) Parent Application or Grant (63) Related by Continuation US 083,542 (CIP) Filed on 7 August 1987 (07.08.87)</p> <p>(71) Applicant (for all designated States except US): GEN-PROBE INCORPORATED [US/US]; 9880 Campus Point Drive, San Diego, CA 92121 (US).</p>	<p>(72) Inventors; and (75) Inventors/Applicants (for US only) : HOGAN, James, John [US/US]; 2727 First Avenue, San Diego, CA 92103 (US). SMITH, Richard, Dana [US/US]; 3522 Mercer Lane, San Diego, CA (US). KOP, Jo, Ann [US/US]; 1314 Hampton Road, San Marcos, CA 92069 (US). McDONOUGH, Sherrol, Hoffa [US/US]; 4697 Robbins Street, San Diego, CA 92122 (US).</p> <p>(74) Agents: KENNEY, J., Ernest; 625 Slaters Lane, Fourth Floor, Alexandria, VA 22314 (US) et al.</p> <p>(81) Designated States: AU, DK, FI, JP, KR, NO, US.</p> <p>Published <i>With international search report.</i></p>																									
<p>(54) Title: NUCLEIC ACID PROBES FOR DETECTION AND/OR QUANTITATION OF NON-VIRAL ORGANISMS</p>																										
<table border="1" style="margin: 10px auto; border-collapse: collapse; text-align: center;"> <caption>Approximate values from the bar chart</caption> <thead> <tr> <th>Organism</th> <th>Value (approx.)</th> </tr> </thead> <tbody> <tr><td>pan-Eubact.</td><td>1000</td></tr> <tr><td>Campylo genus</td><td>450</td></tr> <tr><td>Legion genus</td><td>650</td></tr> <tr><td>Salmon genus</td><td>1100</td></tr> <tr><td>Candida, fungal</td><td>700</td></tr> <tr><td>Mycobact genus</td><td>1050</td></tr> <tr><td>Mycobact sp</td><td>200</td></tr> <tr><td>Chlamydia sp</td><td>150</td></tr> <tr><td>Mycoplasma sp</td><td>450</td></tr> <tr><td>Neisseria sp</td><td>100</td></tr> <tr><td>E. coli-rel</td><td>450</td></tr> </tbody> </table>			Organism	Value (approx.)	pan-Eubact.	1000	Campylo genus	450	Legion genus	650	Salmon genus	1100	Candida, fungal	700	Mycobact genus	1050	Mycobact sp	200	Chlamydia sp	150	Mycoplasma sp	450	Neisseria sp	100	E. coli-rel	450
Organism	Value (approx.)																									
pan-Eubact.	1000																									
Campylo genus	450																									
Legion genus	650																									
Salmon genus	1100																									
Candida, fungal	700																									
Mycobact genus	1050																									
Mycobact sp	200																									
Chlamydia sp	150																									
Mycoplasma sp	450																									
Neisseria sp	100																									
E. coli-rel	450																									
<p>(57) Abstract</p> <p>A method for preparing probes, as well as several probes for use in qualitative or quantitative hybridization assays. The method comprises constructing an oligonucleotide that is sufficiently complementary to hybridize to a region of rRNA selected to be unique to a non-viral organism or group of non-viral organisms sought to be detected, said region of rRNA being selected by comparing one or more variable region rRNA sequences of said non-viral organism or group of non-viral organisms with one or more variable region rRNA sequences from one or more non-viral organisms sought to be distinguished. Hybridization assay probes for <i>Mycobacterium avium</i>, <i>Mycobacterium intracellulare</i>, the <i>Mycobacterium tuberculosis</i>-complex bacteria, <i>Mycoplasma pneumoniae</i>, <i>Legionella</i>, <i>Salmonella</i>, <i>Chlamydia trachomatis</i>, <i>Campylobacter</i>, <i>Proteus mirabilis</i>, <i>Enterococcus</i>, <i>Enterobacter cloacae</i>, <i>E. Coli</i>, <i>Pseudomonas group I</i>, <i>Neisseria gonorrhoeae</i>, bacteria, and fungi also are disclosed.</p>																										

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

S P E C I F I C A T I O N

NUCLEIC ACID PROBES FOR
DETECTION AND/OR QUANTITATION OF
NON-VIRAL ORGANISMS

5 Continuation-In-Part of Hogan et al., App. Ser. No.
083,542 filed August 7, 1987, which is a
Continuation-In-Part of Hogan et al., App. Ser. No.
934,244 filed November 24, 1986.

BACKGROUND OF THE INVENTION

10 1. Field of the Invention

The inventions described and claimed herein relate
to probes and assays based on the use of genetic
material such as RNA. More particularly, the inventions
relate to the design and construction of nucleic acid
15 probes and hybridization of such probes to genetic
material of target non-viral organisms in assays for
detection and/or quantitation thereof in test samples
of, e.g., sputum, urine, blood and tissue sections,
food, soil and water.

20 2. Introduction

Two single strands of nucleic acid, comprised of
nucleotides, may associate ("hybridize") to form a
double helical structure in which the two polynucleotide
chains running in opposite directions are held together
25 by hydrogen bonds (a weak form of chemical bond) between
pairs of matched, centrally located compounds known as
"bases." Generally, in the double helical structure of

nucleic acids, for example, the base adenine (A) is hydrogen bonded to the base thymine (T) or uracil (U) while the base guanine (G) is hydrogen bonded to the base cytosine (C). At any point along the chain, therefore, one may find the base pairs AT or AU, TA or UA, GC, or CG. One may also find AG and GU base pairs in addition to the traditional ("canonical") base pairs. Assuming that a first single strand of nucleic acid is sufficiently complementary to a second and that the two are brought together under conditions which will promote their hybridization, double stranded nucleic acid will result. Under appropriate conditions, DNA/DNA, RNA/DNA, or RNA/RNA hybrids may be formed.

Broadly, there are two basic nucleic acid hybridization procedures. In one, known as "in solution" hybridization, both a "probe" nucleic acid sequence and nucleic acid molecules from a test sample are free in solution. In the other method, the sample nucleic acid is usually immobilized on a solid support and the probe sequence is free in solution.

A probe may be a single strand nucleic acid sequence which is complementary in some particular degree to the nucleic acid sequences sought to be detected ("target sequences"). It may also be labelled. A background description of the use of nucleic acid hybridization as a procedure for the detection of particular nucleic acid sequences is described in U.S. Application Serial No. 456,729, entitled "Method for Detection, Identification and Quantitation of Non-Viral Organisms," filed January 10, 1983 (Kohne I), and U.S. Application Serial No. 655,365, entitled "Method For Detecting, Identifying and Quantitating Organisms and Viruses," filed September 4, 1984 (Kohne II), both of

which are incorporated by reference, together with all other applications cited herein.

Also described in those applications are methods for determining the presence of RNA-containing organisms in a sample which might contain such organisms, comprising the steps of bringing together any nucleic acids from a sample and a probe comprising nucleic acid molecules which are shorter than the rRNA subunit sequence from which it was derived and which are sufficiently complementary to hybridize to the rRNA of one or more non-viral organisms or groups of non-viral organisms, incubating the mixture under specified hybridization conditions, and assaying the resulting mixture for hybridization of the probe and any test sample rRNA. The invention is described to include using a probe which detects only rRNA subunit subsequences which are the same or sufficiently similar in particular organisms or groups of organisms and is said to detect the presence or absence of any one or more of those particular organisms in a sample, even in the presence of many non-related organisms.

We have discovered and describe herein a novel method and means for designing and constructing DNA probes for use in detecting unique rRNA sequences in an assay for the detection and/or quantitation of any group of non-viral organisms. Some of the inventive probes herein may be used to detect and/or quantify a single species or strain of non-viral organism and others may be used to detect and/or quantify members of an entire genus or desired phylogenetic grouping.

SUMMARY OF THE INVENTION

In a method of probe preparation and use, a single strand deoxyoligonucleotide of particular sequence and defined length is used in a hybridization assay to determine the presence or amount of rRNA from particular target non-viral organisms to distinguish them from their known closest phylogenetic neighbors. Probe sequences which are specific, respectively, for 16S rRNA variable subsequences of Mycobacterium avium, Mycobacterium intracellulare and the Mycobacterium tuberculosis-complex bacteria, and which do not cross react with nucleic acids from each other, or any other bacterial species or respiratory infectious agent, under proper stringency, are described and claimed. A probe specific to three 23S rRNA variable region subsequences from the Mycobacterium tuberculosis-complex bacteria is also described and claimed, as are rRNA variable region probes useful in hybridization assays for the genus Mycobacterium (16S 23S rRNA specific), Mycoplasma pneumoniae (5S and 16S rRNA-specific), Chlamydia trachomatis (16S and 23S rRNA specific), Enterobacter cloacae (23S rRNA specific), Escherichia coli (16S rRNA specific), Legionella (16S and 23S rRNA specific), Salmonella (16S and 23S rRNA specific), Enterococci (16S rRNA specific), Neisseria gonorrhoeae (16s rRNA specific), Campylobacter (16S rRNA specific), Proteus mirabilis (23S rRNA specific), Pseudomonas (23S rRNA specific), fungi (18S and 28S rRNA specific), and bacteria (16S and 23S rRNA specific).

In one embodiment of the assay method, a test sample is first subjected to conditions which release rRNA from any non-viral organisms present in that sample. rRNA is single stranded and therefore available for hybridization with sufficiently complementary

genetic material once so released. Contact between a probe, which can be labelled, and the rRNA target may be carried out in solution under conditions which promote hybridization between the two strands. The reaction mixture is then assayed for the presence of hybridized probe. Numerous advantages of the present method for the detection of non-viral organisms over prior art techniques, including accuracy, simplicity, economy and speed will appear more fully from the detailed description which follows.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a chart of the primary structure of bacterial 16S rRNA for Escherichia coli, depicting standard reference numbers for bases.

Figure 2 is a chart of the primary structure of bacterial 23S rRNA for Escherichia coli, depicting standard reference numbers for bases.

Figure 3 is a chart of the primary structure of bacterial 5S rRNA for Escherichia coli, depicting standard reference numbers for bases.

Figure 4 is a chart of the primary structure for the 18S rRNA for Saccharomyces cerevisiae, depicting standard reference numbers for bases.

Figure 5 is a chart of the primary structure for the 28S rRNA for Saccharomyces cerevisiae, depicting standard reference numbers for bases.

Figure 6 is a diagram showing the locations in the 16S rRNA (using E. coli reference numbers) which differ between 12 different sets of related organisms. In Example 1, for example, 99.7 refers to the difference in 16s rRNA between Clostridium botulinum and Clostridium subterminale.

Figure 7 is a diagram showing the locations in the first 1500 bases of 23S rRNA (using E.coli reference numbers) which differ between 12 different sets of related organisms.

5 Figure 8 is a diagram showing the locations in the terminal bases of 23S rRNA (using E.coli reference numbers) which differ between 12 different sets of related organisms.

10 Figure 9 is a schematic representation of the location of probes capable of hybridizing to the 16S rRNA.

Figure 10 is a schematic representation of the location of probes capable of hybridizing to the first 1500 bases of the 23S rRNA.

15 Figure 11 is a schematic representation of the location of probes capable of hybridizing to the terminal bases of 23S rRNA.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

20 The following terms, as used in this disclosure and claims, are defined as:

nucleotide: a subunit of a nucleic acid consisting of a phosphate group, a 5' carbon sugar and a nitrogen containing base. In RNA the 5' carbon sugar is
25 ribose. In DNA, it is a 2-deoxyribose. The term also includes analogs of such subunits.

nucleotide polymer: at least two nucleotides linked by phosphodiester bonds.

oligonucleotide: a nucleotide polymer generally
30 about 10 to about 100 nucleotides in length, but which may be greater than 100 nucleotides in length.

nucleic acid probe: a single stranded nucleic acid sequence that will combine with a complementary single stranded target nucleic acid sequence to form a double-stranded molecule (hybrid). A nucleic acid probe
5 may be an oligonucleotide or a nucleotide polymer.

hybrid: the complex formed between two single stranded nucleic acid sequences by Watson-Crick base pairings or non-canonical base pairings between the complementary bases.

10 hybridization: the process by which two complementary strands of nucleic acids combine to form double stranded molecules (hybrids).

complementarity: a property conferred by the base sequence of a single strand of DNA or RNA which may
15 form a hybrid or double stranded DNA:DNA, RNA:RNA or DNA:RNA through hydrogen bonding between Watson-Crick base pairs on the respective strands. Adenine (A) usually complements thymine (T) or Uracil (U), while guanine (G) usually complements cytosine (C).

20 stringency: term used to describe the temperature and solvent composition existing during hybridization and the subsequent processing steps. Under high stringency conditions only highly homologous nucleic acid hybrids will form; hybrids without a sufficient
25 degree of complementarity will not form. Accordingly, the stringency of the assay conditions determine the amount of complementarity needed between two nucleic acid strands forming a hybrid. Stringency is chosen to maximize the difference in stability between the hybrid
30 formed with the target and the nontarget nucleic acid.

probe specificity: characteristic of a probe which describes its ability to distinguish between target and non-target sequences. Dependent on sequence and assay conditions. Probe specificity may be absolute
35 (i.e., probe able to distinguish between target

organisms and any nontarget organisms), or it may be functional (i.e., probe able to distinguish between the target organism and any other organism normally present in a particular sample). Many probe sequences can be
5 used for either broad or narrow specificity depending on the conditions of use.

variable region: nucleotide polymer which differs by at least one base between the target organism and nontarget organisms contained in a sample.

10 conserved region: a region which is not variable.

sequence divergence: process by which nucleotide polymers become less similar during evolution.

15 sequence convergence: process by which nucleotide polymers become more similar during evolution.

bacteria: members of the phylogenetic group eubacteria, which is considered one of the three primary kingdoms.

20 T_m: temperature at which 50% of the probe is converted from the hybridized to the unhybridized form.

thermal stability: Temperature at which 50% of the probe:target hybrids are converted to the single stranded form. Factors which affect the thermal
25 stability can affect probe specificity and therefore, must be controlled. Whether a probe sequence is useful to detect only a specific type of organism depends largely on the thermal stability difference between probe:target hybrids ("P:T") and probe:nontarget hybrids
30 ("P:NT"). In designing probes the T_m P:T minus the T_m P:NT should be as large as possible.

In addition to a novel method for selecting probe sequences, we have discovered that it is possible to create a DNA probe complementary to a particular rRNA
35 sequence obtained from a single type of target

microorganism and to successfully use that probe in a non-cross reacting assay for the detection of that single microorganism, even in the presence of its known, most closely related taxonomic or phylogenetic neighbors. With the exception of viruses, all prokaryotic organisms contain rRNA molecules including 5S rRNA, 16S rRNA, and a larger rRNA molecule known as 23S rRNA. Eukaryotes are known to have 5.0S, 5.8S, 18S and 28S rRNA molecules or analogous structures. (The term "16S like" sometimes is used to refer to the rRNA found in the small ribosomal subunit, including 18S and 17S rRNA. Likewise the term "23S like" rRNA sometimes is used to refer to the rRNA found in the large ribosomal subunit. 5.8S rRNA is equivalent to the 5' end of the 23S like rRNA.) These rRNA molecules contain nucleotide sequences which are highly conserved among all organisms thus far examined. There are known methods which allow a significant portion of these rRNA sequences to be determined. For example, complementary oligonucleotide primers of about 20-30 bases in length can be hybridized to universally conserved regions in purified rRNA that are specific to the 5S, 16S, or 23S subunits and extended with the enzyme reverse transcriptase. Chemical degradation or dideoxynucleotide- terminated sequencing reactions can be used to determine the nucleotide sequence of the extended product. Lane, D.J. et al., Proc. Nat'l Acad. Sci. USA 82, 6955-6959 (1985).

In our invention, comparison of one or more sequenced rRNA variable regions from a target organism to one or more rRNA variable region sequences from a closely related bacterial species is utilized to select a sequence unique to the rRNA of the target organism. rRNA is preferable to DNA as a probe target because of

its relative abundance and stability in the cell and because of its patterns of phylogenetic conservation.

Notwithstanding the highly conserved nature of rRNA, we have discovered that a number of regions of the rRNA molecule which can vary in sequence, can vary even between closely related species and can, therefore, be utilized to distinguish between such organisms. Differences in the rRNA molecule are not distributed randomly across the entire molecule, but rather are clustered into specific regions. The degree of conservation also varies, creating a unique pattern of conservation across the ribosomal RNA subunits. The degree of variation and the distribution thereof, can be analyzed to locate target sites for diagnostic probes. This method of probe selection may be used to select more than one sequence which is unique to the rRNA of a target organism.

We have identified variable regions by comparative analysis of rRNA sequences both published in the literature and sequences which we have determined ourselves using procedures known in the art. We use a Sun Microsystems (TM) computer for comparative analysis. The compiler is capable of manipulating many sequences of data at the same time. Computers of this type and computer programs which may be used or adapted for the purposes herein disclosed are commercially available.

Generally, only a few regions are useful for distinguishing between closely related species of a phylogenetically conserved genus, for example, the region 400-500 bases from the 5' end of the 16S rRNA molecule. An analysis of closely related organisms (Figures 6, 7 and 8) reveals the specific positions (variable regions) which vary between closely related organisms. These variable regions of rRNA molecules are the likely candidates for probe design.

Figures 5, 6 and 7 display the variations in 16S and 23S rRNA's between two different bacteria with decreasing amounts of similarity between them. Closer analysis of these figures reveals some subtle patterns between these closely related organisms. In all cases studied, we have seen sufficient variation between the target organism and the closest phylogenetic relative found in the same sample to design the probe of interest. Moreover, in all cases studied to date, the per cent similarity between the target organism (or organisms) and the closest phylogenetically related organisms found in the same sample has been between 90% and 99%. Interestingly, there was enough variation even between the rRNA's of *Neisseria's gonorrhoeae* and *meningitidis* (See Example 21) to design probes - despite the fact that DNA:DNA homology studies suggested these two species might actually be one and the same.

These figures also show that the differences are distributed across the entire 16S and 23S rRNA's. Many of the differences, nonetheless, cluster into a few regions. These locations in the rRNA are good candidates for probe design, with our current assay conditions. We also note that the locations of these increased variation densities usually are situated in the same regions of the 16S and 23S rRNA for comparable per cent similarity values. In this manner, we have observed that certain regions of the 16S and 23S rRNA are the most likely sites in which significant variation exists between the target organism and the closest phylogenetic relatives found in a sample. We have disclosed and claimed species specific probes which hybridize in these regions of significant variation between the target organism and the closest phylogenetic relative found in a sample.

Figures 9, 10 and 11 are a schematic representation of the location of probes disclosed and claimed herein. Because 16S and 23S RNAs do not, as a rule, contain sequences of duplication longer than about
5 six nucleotides in length, probes designed by these methods are specific to one or a few positions on the target nucleic acid.

The sequence evolution at each of the variable regions (for example, spanning a minimum of 10
10 nucleotides) is, for the most part divergent, not convergent. Thus, we can confidently design probes based on a few rRNA sequences which differ between the target organism and its phylogenetically closest relatives. Biological and structural constraints on the
15 rRNA molecule which maintain homologous primary, secondary and tertiary structure throughout evolution, and the application of such constraints to probe diagnostics is the subject of ongoing study. The greater the evolutionary distance between organisms, the
20 greater the number of variable regions which may be used to distinguish the organisms.

Once the variable regions are identified, the sequences are aligned to reveal areas of maximum
25 homology or "match". At this point, the sequences are examined to identify potential probe regions. Two important objectives in designing a probe are to maximize homology to the target sequence(s) (greater than 90% homology is recommended) and to minimize
30 homology to non-target sequence(s) (less than 90% homology to nontargets is recommended). We have identified the following useful guidelines for designing probes with desired characteristics.

First, probes should be positioned so as to
35 minimize the stability of the probe:nontarget nucleic

acid hybrid. This may be accomplished by minimizing the length of perfect complementarity to non-target organisms, avoiding G and C rich regions of homology to non-target sequences, and by positioning the probe to span as many destabilizing mismatches as possible (for example, dG:rU base pairs are less destabilizing than some others).

Second, the stability of the probe: target nucleic acid hybrid should be maximized. This may be accomplished by avoiding long A and T rich sequences, by terminating the hybrids with G:C base pairs and by designing the probe with an appropriate T_m . The beginning and end points of the probe should be chosen so that the length and %G and %C result in a T_m about 2-10°C higher than the temperature at which the final assay will be performed. The importance and effect of various assay conditions will be explained further herein. Third, regions of the rRNA which are known to form strong structures inhibitory to hybridization are less preferred. Finally, probes with extensive self-complementarity should be avoided.

In some cases, there may be several sequences from a particular region which will yield probes with the desired hybridization characteristics. In other cases, one sequence may be significantly better than another which differs merely by a single base.

The following chart indicates how, for one embodiment of the invention useful in the detection of a nucleic acid in the presence of closely related nucleic acid sequences, unique sequences can be selected. In this example, rRNA sequences have been determined for organisms A-E and their sequences, represented numerically, are aligned as shown. It is seen that sequence 1 is common to all organisms A-E. Sequences 2-6 are found only in organisms A, B and C, while

sequences 8, 9 and 10 are unique to organism A. Therefore, a probe complementary to sequences 8, 9 or 10 would specifically hybridize to organism A.

5

Illustrative Pattern of Sequence
Relationships Among Related Bacteria

<u>Organism</u>		<u>rRNA Sequence</u>									
10	A	1	2	3	4	5	6	7	8	9	10
	B	1	2	3	4	5	6	7	11	12	13
	C	1	2	3	4	5	6	14	15	16	17
	D	1	18	19	20	21	22	23	24	25	26
	E	1	18	19	20	21	27	28	29	30	31

In cases where the patterns of variation of a macromolecule are known, for example, rRNA, one can focus on specific regions as likely candidates for probe design. However, it is not always necessary to determine the entire nucleic acid sequence in order to obtain a probe sequence. Extension from any single oligonucleotide primer can yield up to 300-400 bases of sequence. When a single primer is used to partially sequence the rRNA of the target organism and organisms closely related to the target, an alignment can be made as outlined above. Plainly, if a useful probe sequence is found, it is not necessary to continue rRNA sequencing using other primers. If, on the other hand, no useful probe sequence is obtained from sequencing with a first primer, or if higher sensitivity is desired, other primers can be used to obtain more sequences. In those cases where patterns of variation

for a molecule are not well understood, more sequence data may be required prior to probe design.

Thus, in Examples 1-3 below, two 16S-derived primers were used. The first primer did not yield probe
5 sequences which met the criteria listed herein. The second primer yielded probe sequences which were determined to be useful following characterization and testing for specificity as described. In Example 4, six 23S primers were used prior to locating the probe
10 sequence set forth.

Once a presumptive unique sequence has been identified, a complementary DNA oligonucleotide is synthesized. This single stranded oligonucleotide will serve as the probe in the DNA/rRNA assay hybridization
15 reaction. Defined oligonucleotides may be synthesized by any of several well known methods, including automated solid-phase chemical synthesis using cyanoethylphosphoramidite precursors. Barone, A.D. et al., Nucleic Acids Research 12, 4051-4060 (1984). In this
20 method, deoxyoligonucleotides are synthesized on solid polymer supports. Release of the oligonucleotide from the support is accomplished by treatment with ammonium hydroxide at 60°C for 16 hours. The solution is dried and the crude product is dissolved in water and
25 separated on polyacrylamide gels which generally may vary from 10-20% depending upon the length of the

fragment. The major band, which is visualized by ultraviolet back lighting, is cut from the gel with a razor blade and extracted with 0.1M ammonium acetate, pH 7.0, at room temperature for 8-12 hours. Following
5 centrifugation, the supernatant is filtered through a 0.4 micron filter and desalted on a P-10 column (Pharmacia). Other well known methods for construction of synthetic oligonucleotides may, of course, be employed.

10 Current DNA synthesizers can produce large amounts of synthetic DNA. After synthesis, the size of the newly made DNA is examined by gel filtration and molecules of varying size are generally detected. Some of these molecules represent abortive synthesis events
15 which occur during the synthesis process. As part of post-synthesis purification, the synthetic DNA is usually size fractionated and only those molecules which are the proper length are kept. Thus, it is possible to obtain a population of synthetic DNA molecules of
20 uniform size.

It has been generally assumed, however, that synthetic DNA is inherently composed of a uniform population of molecules all of the same size and base sequence, and that the hybridization characteristics of
25 every molecule in the preparation should be the same. In reality, preparations of synthetic DNA molecules are

heterogeneous and are composed of significant numbers of molecules which, although the same size, are in some way different from each other and have different hybridization characteristics. Even different
5 preparations of the same sequence can sometimes have different hybridization characteristics.

Accordingly, preparations of the same synthetic probe sequence can have different hybridization characteristics. Because of this the specificity of probe
10 molecules from different preparations can be different. The hybridization characteristics of each preparation should be examined in order to determine the hybridization conditions which must be used in order to obtain the desired probe specificity. For example, the
15 synthetic probe described in Example 4 below has the specificity profile described in Table 14. This data was obtained by using the hybridization and assay conditions described. A separate preparation of this probe which has different
20 hybridization characteristics may not have precisely the same specificity profile when assayed under the conditions presented in Example 4. Such probe preparations have been made. To obtain the desired specificity, these probes can be hybridized and assayed
25 under different conditions, including salt concentration and/or temperature. The actual conditions under which

SUBSTITUTE SHEET

the probe is to be used must be determined, or matched to extant requirements, for each batch of probe since the art of DNA synthesis is somewhat imperfect.

Following synthesis and purification of a particular oligonucleotide sequence, several procedures may be utilized to determine the acceptability of the final product. The first is polyacrylamide gel electrophoresis, which is used to determine size. The oligonucleotide is labelled using, for example, ^{32}P -ATP and T_4 polynucleotide kinase. The labelled probe is precipitated in ethanol, centrifuged and the dried pellet resuspended in loading buffer (80% formamide, 20 mM NaOH, 1 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol). The samples are heated for five minutes at 90°C and loaded onto a denaturing polyacrylamide gel. Electrophoresis is carried out in TBE buffer (0.1 M Tris HCl pH 8.3, 0.08 M boric acid, 0.002 M EDTA) for 1-2 hours at 1,000 volts. Following electrophoresis of the oligonucleotide the gel is exposed to X-ray film. The size of the oligonucleotide is then computed from the migration of oligonucleotide standards run concurrently.

The sequence of the synthetic oligonucleotide may also be checked by labelling it at the 5' end with ^{32}P -ATP and T_4 polynucleotide kinase, subjecting it to standard chemical degradation techniques, Maxam, A.M. and Gilbert, W., Proc. Nat'l. Acad. Sci. USA 74, 560-564

(1980), and analyzing the products on polyacrylamide gels. Preferably, the nucleotide sequence of the probe is perfectly complementary to the previously identified unique rRNA sequence, although it need not be.

5 The melting profile, including the melting temperature (T_m) of the oligonucleotide/ rRNA hybrids should also be determined. One way to determine T_m is to hybridize a ^{32}P -labelled oligonucleotide to its complementary target nucleic acid at 50°C in 0.1 M
10 phosphate buffer, pH 6.8. The hybridization mixture is diluted and passed over a 2cm hydroxyapatite column at 50°C . The column is washed with 0.1 M phosphate buffer, 0.02% SDS to elute all unhybridized, single-stranded probes. The column temperature is then dropped 15°C and
15 increased in 5°C increments until all of the probe is single-stranded. At each temperature, unhybridized probe is eluted and the counts per minute (cpm) in each fraction determined. The number of cpm shown to be bound to the hydroxyapatite divided by the total cpm
20 added to the column equals the percent hybridization of the probe to the target nucleic acid.

 An alternate method for determining thermal stability of a hybrid is outlined below. An aliquot of hybrid nucleic acid is diluted into 1 ml of either 0.12M
25 phosphate buffer, 0.2% SDS, 1mM EDTA, 1mM EGTA or an appropriate hybridization buffer. Heat this 1 ml of

SUBSTITUTE SHEET

solution to 45 degrees C for 5 minutes and place it into a room temperature water bath to cool for 5 minutes. Assay this 1 ml of hybrid containing solution over a hydroxyapatite column, capturing the hybrid and washing away unbound probe. If a hybridization solution other than the 0.12M phosphate buffer is used, then a dilution of the hybridization solution into the 0.12M phosphate buffer will be necessary for binding. Keep taking aliquots of hybrid and diluting into 1 ml of hybridization solution or into the standard 0.12M phosphate buffer solution described above while raising the heating temperature 5 degrees C at a time. Continue this until all of the hybrid is dissociated. The point where one half of the hybrid is converted to the dissociated form is considered the T_m . The T_m for a given hybrid will vary depending on the hybridization solution being used because the thermal stability depends upon the concentration of different salts, detergents, and other solutes which effect relative hybrid stability during thermal denaturation.

Because the extent and specificity of hybridization reactions such as those described herein are affected by a number of factors, manipulation of one or more of those factors will determine the exact sensitivity and specificity of a particular probe, whether perfectly complementary to its target or not.

SUBSTITUTE SHEET

For example, the base composition of the probe may be significant because G-C base pairs exhibit greater thermal stability as compared to A-T base pairs due to additional hydrogen bonding.

- 5 Thus, hybridization involving complementary nucleic acids of higher G-C content will be stable at higher temperatures.

We have discovered that the length of the target nucleic acid sequence and, accordingly, the length of
10 the probe sequence can also be important. While it is possible for nucleic acids that are not perfectly complementary to hybridize, the longest stretch of perfectly homologous base sequence will normally primarily determine hybrid stability. While
15 oligonucleotide probes of different lengths and base composition may be used, oligonucleotide probes preferred in this invention are between about 15 and about 50 bases in length and are at least about 75-100% homologous to the target nucleic acid. For most
20 applications 95-100% homology to the target nucleic acid is preferred.

Ionic strength and incubation temperature should also be taken into account in constructing a probe. It is known that the rate of hybridization will increase as
25 ionic strength of the reaction mixture increases and that the thermal stability of hybrids will increase with

increasing ionic strength. In general, optimal hybridization for synthetic oligonucleotide probes of about 15-50 bases in length occurs approximately 5°C below the melting temperature for a given duplex.

5 Incubation at temperatures below the optimum may allow mismatched base sequences to hybridize and can therefore result in reduced specificity.

As to nucleic acid concentration, it is known that the rate of hybridization is proportional to the
10 concentration of the two interacting nucleic acid species. Thus, the presence of compounds such as dextran and dextran sulphate are thought to increase the local concentration of nucleic acid species and thereby result in an increased rate of hybridization. Other
15 agents which will result in increased rates of hybridization are specified in U.S. Application Serial No. 627,795, entitled "Accelerated Nucleic Acid Reassociation Method", filed July 5, 1984, Continuation-in-Part thereof, Serial No. (not yet assigned), filed
20 June 4, 1987, and U.S. Application Serial No. 816,711, entitled "Accelerated Nucleic Acid Reassociation Method", filed January 7, 1986, both of which are incorporated by reference. On the other hand, chemical reagents which disrupt hydrogen bonds such as formamide,
25 urea, DMSO, and alcohols will increase the stringency of hybridization.

Selected oligonucleotide probes may be labelled by any of several well known methods. Useful labels include radioisotopes as well as non-radioactive reporting groups. Isotopic labels include ^3H , ^{35}S , ^{32}P , ^{125}I , Cobalt and ^{14}C . Most methods of isotopic labelling involve the use of enzymes and include the known methods of nick translation, end labelling, second strand synthesis, and reverse transcription. When using radio-labelled probes, hybridization can be detected by autoradiography, scintillation counting, or gamma counting. The detection method selected will depend upon the hybridization conditions and the particular radioisotope used for labelling.

Non-isotopic materials can also be used for labelling, and may be introduced by the incorporation of modified nucleotides through the use of enzymes or by chemical modification of the probe, for example, by the use of non-nucleotide linker groups. Non-isotopic labels include fluorescent molecules, chemiluminescent molecules, enzymes, cofactors, enzyme substrates, haptens or other ligands. We currently prefer to use acridinium esters.

In one embodiment of the DNA/rRNA hybridization assay invention, a labelled probe and bacterial target nucleic acids are reacted in solution. rRNA may be released from bacterial cells by the sonic disruption

method described in Murphy, K.A. et al., U.S. Application Serial No. 841,860, entitled "Method for Releasing RNA and DNA From Cells", filed March 20, 1986, which is incorporated herein by reference. Other known
5 methods for disrupting cells include the use of enzymes, osmotic shock, chemical treatment, and vortexing with glass beads. Following or concurrent with the release of rRNA, labelled probe may be added in the presence of accelerating agents and incubated at the optimal
10 hybridization temperature for a period of time necessary to achieve significant reaction. Following this incubation period, hydroxyapatite may be added to the reaction mixture to separate the probe/rRNA hybrids from the non-hybridized probe molecules. The hydroxyapatite
15 pellet is washed, recentrifuged and hybrids detected by means according to the label used.

Twenty-one embodiments illustrative of the claimed inventions are set forth below, in which a synthetic probe or probes complementary to a unique rRNA sequence
20 from a target organism, or group of organisms is determined, constructed and used in a hybridization assay.

DESCRIPTION OF PARTICULAR EMBODIMENTS

Mycobacterium are acid-fast, alcohol fast,
25 aerobic, non-mobile bacilli. Their lipid content is

high and their growth slow. Mycobacterium avium and Mycobacterium intracellulare are together referred to as M. avium-intracellulare because they are so difficult to differentiate. Recently, the M. avium complex, which
5 includes M. intracellulare, was shown to be the second most commonly isolated, clinically significant Mycobacterium. Good, R.C. et al., J. Infect. Dis. 146, 829-833 (1982). More recent evidence indicates that these organisms are a
10 common cause of opportunistic infection in patients with AIDS (acquired immune deficiency syndrome). Gill, V.J. et al., J. Clin. Microbio. 22, 543-546 (1985). Treatment of such infections in AIDS patients is difficult because these organisms are resistant to most
15 antituberculosis drugs. Often a combination of five drugs are used in therapy. The severity of these infections also requires rapid diagnosis which, prior to the invention herein, was not available.

Members of the Mycobacterium tuberculosis complex
20 (Mtb) include Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum and Mycobacterium microti. The first three are pathogenic for humans while the last is an animal pathogen. These organisms produce slowly developing granulomas on the skin or they
25 may invade internal organs. Tuberculosis of the lungs can be disseminated to other parts of the body by the

circulatory system, the lymph system, or the intestinal tract. Despite advances in public health and the advent of effective chemotherapy, Mycobacterial disease, tuberculosis in particular, continues to represent a
5 major world-wide health problem.

The classical method for detecting bacteria in a test sample involves culturing of the sample in order to expand the number of bacterial cells present into observable colony growths which can be identified and
10 enumerated. If desired, the cultures can also be subjected to additional testing in order to determine antimicrobial susceptibility. Currently, the most widely used procedures for the detection, isolation and identification of Mycobacterium species are the acid-
15 fast bacilli (AFB) smear (using either the Ziehl-Neelsen or fluorochrome techniques), culture methods using Lowenstein-Jensen media and Middlebrook media, and biochemical tests. The AFB relies on the high lipid content of Mycobacterium to
20 retain dye after exposure to acid- alcohol. While the AFB smear test is relatively rapid and simple to perform it does not always detect Mycobacteria and will not differentiate between Mycobacterium avium and non-tuberculosis species, between Mycobacterium
25 intracellulare and non- tuberculosis species, or between Mycobacterium tuberculosis- complex bacilli and non-

SUBSTITUTE SHEET

CGK00033161

tuberculosis species. For accurate identification of the infecting Mycobacterial species the clinician must rely on culture results which can require anywhere from 3 to 8 weeks of growth followed by extensive biochemical testing. Other tests have been developed based on the detection of metabolic products from Mycobacterium using carbon-14 labelled substrates. In particular, the Bactec (TM) instrument can detect the presence of Mycobacterium within 6 to 10 days of the time of inoculation. Gill, V.J., supra. However, the test does not distinguish Mycobacterium species. It is often important to make this determination so that particular drugs to which the organism is susceptible may be prescribed. For traditional culture methods, this requires an additional 2 to 3 weeks and for the Bactec method, an additional 6 to 10 days.

In addition, specific embodiments for Mycoplasma pneumoniae, the Mycobacterium, Legionella, Salmonella, Chlamydia trachomatis, Campylobacter, Proteus mirabilis, Enterococcus, Enterobacter cloacae, E. coli, Pseudomonas Group I, bacteria, fungi and Neisseria gonorrhoeae are set forth in the following examples.

As indicated by the below examples, the present invention has significant advantages over each of these prior art methods not only in the enhanced accuracy, specificity and simplicity of the test, but also in

greatly reducing the time to achieve a diagnosis. The invention makes possible a definitive diagnosis and initiation of effective treatment on the same day as testing.

5

Example 1

Described below is the preparation of a single strand deoxyoligonucleotide of unique sequence and defined length which is labelled and used as a probe in a solution hybridization assay to detect the presence of

10 rRNA from Mycobacterium avium. This unique sequence is specific for the rRNA of Mycobacterium avium and does not significantly cross-react under the hybridization conditions of this Example, with nucleic acids from any other bacterial species or respiratory infectious agent,

15 including the closely-related Mycobacterium intracellulare. This probe is able to distinguish the two species, notwithstanding an approximate 98% rRNA homology between the two species. In this Example, as well as in Examples 2 and 3, sequences for M. avium, M.

20 tuberculosis complex, M. intracellulare and related organisms were obtained by using a specific primer to a highly conserved region in the 16S rRNA. The sequence of this primer, derived from E. coli rRNA, was 5'-GGC CGT TAC CCC ACC TAC TAG CTA AT-3'. 5 nanograms of

25 primer was mixed with 1 microgram of each rRNA to be

sequenced in the presence of 0.1M KCl and 20mM Tris-HCl
pH 8.3 in a final volume of 10 microliters. The
reactions were heated 10 min. at 45°C and then placed on
ice. 2.5 microliters of ³⁵S dATP and 0.5 microliters of
5 reverse transcriptase were added. The sample was
aliquoted into 4 tubes, each tube containing either
dideoxy A, G, T, or C. The concentrations of these
nucleotides are set forth in Lane et al., supra. The
samples were incubated at 40°C for 30 minutes, and were
10 then precipitated in ethanol, centrifuged and the
pellets lyophilized dry. Pellets were resuspended in 10
microliters formamide dyes (100% formamide, 0.1%
bromphenol blue and 0.1% xylene cyanol), and loaded onto
80 cm 8% polyacrylamide gels. The gels were run at
15 2000 volts for 2-4 hours.

Thus, nucleotide sequences for the 16S rRNA of
Mycobacterium avium and what were considered to be its
closest phylogenetic neighbors, Mycobacterium
intracellulare and Mycobacterium tuberculosis, were
20 determined by the method of Lane, D.J. et al., Proc.
Nat. Acad. Sci. USA 82:6955 (1985). In addition to
determining the rRNA sequences for the organisms noted
above, a spectrum of clinically significant
Mycobacterium were also sequenced. These included M.
25 fortuitum, M. scrofulaceum and M. chelonae. Selected
members of several genera closely related to

Mycobacterium were also sequenced, including Rhodococcus bronchialis, Corynebacterium xerosis and Nocardia asteroides.

Partial rRNA sequences from the above organisms were aligned for maximum nucleotide homology, using commercially available software from Intelligenetics, Inc., 1975 El Camino Real West, Mountain View, California 94040-2216 (IFIND Program). From this alignment, regions of sequence unique to Mycobacterium avium were determined. The probe was selected so that it was perfectly complementary to a target nucleic acid sequence and so that it had a 10% or greater mismatch with the aligned rRNA from its known closest phylogenetic neighbor. A sequence 38 bases in length was chosen. The number of mismatched bases relative to the Mycobacterium avium sequence were as follows: Mycobacterium tuberculosis (8); Mycobacterium intracellulare (5); Mycobacterium scrofulaceum (6); Mycobacterium chelonae (12); and Mycobacterium fortuitum (10).

The following cDNA sequence was characterized by the criteria of length, T_m, and sequence analysis as described at pages 7-8 above and was determined to be specific for the rRNA

Mycobacterium avium:

ACCGCAAAAGCTTTCCACCAGAAGACATGCGTCTTGAG.

SUBSTITUTE SHEET

This sequence is complementary to a unique segment found in the 16S rRNA of Mycobacterium avium. The size of the probe is 38 bases. The probe has a T_m of 74°C and sequence analysis by the method of Maxam & Gilbert
5 (1980), supra, confirmed that the probe was correctly synthesized. The probe is capable of hybridizing to rRNA of M. avium in the region corresponding to bases 185-225 of E. coli 16S rRNA.

To demonstrate the reactivity of this sequence for
10 Mycobacterium avium, it was tested as a probe in hybridization reactions under the following conditions. ^{32}P -end-labeled oligonucleotide probes were mixed with 1 microgram (7×10^{-13} moles) of purified rRNA from Mycobacterium avium and reacted in 0.12 M PB
15 hybridization buffer (equimolar amounts of Na_2HPO_4 and NaH_2PO_4), 1 mM EDTA and 0.02% SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was mixed with the hybridization buffer both with and without target
20 present. Following separation on hydroxyapatite as outlined in the patent applications identified at page 2, supra, the hybrids were quantitated by scintillation counting. These results are presented in Table 1, showing that the probe has a high extent of reaction to

homologous target and very little non-specific binding to the hydroxyapatite.

TABLE 1

HYBRIDIZATION OF THE *M. AVIUM* PROBE
TO HOMOLOGOUS TARGET rRNA*

	<u>plus rRNA</u>	<u>minus rRNA</u>
<i>M. avium</i> probe	85-95%	0.5%
10 * % Hybridization =	$\frac{\text{cpm bound to hydroxyapatite}}{\text{total cpm added to reaction}}$	

Specificity of the probe for *M. avium* was tested by
 15 mixing the ^{32}P labeled probe with rRNA released from
 cells of 29 other species of mycobacteria by the sonic
 disruption techniques described in Murphy et al., U.S.
 Application Serial No. 841,860. 1×10^8 cells were
 20 suspended in 0.1 ml 5% SDS and sonicated for 10 minutes
 at 50-60°C. 1.0 ml of hybridization buffer (45% sodium
 diisobutyl sulfosuccinate, 40 mM phosphate buffer pH 6.8
 and 1 mM EDTA) was added and the mixture incubated for
 60 minutes at 72°C. Following incubation, 4.0 ml of
 25 hydroxyapatite solution (0.14M sodium phosphate buffer,
 pH 6.8, 0.02% SDS and 1.0 gram hydroxyapatite per 50 mls
 solution) was added and incubated for 5 minutes at 72°C.
 The sample was centrifuged and the supernatant removed.
 4.0 ml wash solution (0.14 M sodium phosphate pH 6.8)
 was added and sample was vortexed, centrifuged and the
 30 supernatant removed. The radioactivity bound to the
 hydroxyapatite was determined by scintillation counting.
 The results are shown in Table 2 and indicate that the

SUBSTITUTE SHEET

probe is specific for Mycobacterium avium and does not react with any other mycobacterial species, including Mycobacterium intracellulare.

5

TABLE 2

HYBRIDIZATION OF THE M. AVIUM PROBE TO
MYCOBACTERIAL SPECIES

10	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Mycobacterium africanum	25420	1.0
	M. asiaticum	25276	1.2
	M. avium	25291	87.6
	M. bovis	19210	1.2
15	M. bovis (BCG)	19015	1.0
	M. chelonae	14472	0.9
	M. flavescens	14474	0.9
	M. fortuitum	6841	1.0
	M. gastri	15754	1.2
20	M. gordonae	14470	1.2
	M. haemophilum	29548	1.3
	M. intracellulare	13950	1.5
	M. kansasii	12478	1.2
	M. malmoense	29571	1.2
25	M. marinum	827	1.2
	M. nonchromogenicum	1930	1.1
	M. phlei	11758	1.3
	M. scrofulaceum	19981	1.2
	M. shimoides	27962	2.3
30	M. simiae	25275	1.2
	M. smegmatis	el4468	1.0
	M. szulgai	23069	1.0
	M. terrae	15755	1.2
	M. thermoresistibile	19527	1.3
35	M. triviale	23292	1.2
	M. tuberculosis (avirulent)	25177	1.4
	M. tuberculosis (virulent)	27294	1.1
	M. ulcerans	19423	1.4
	M. vaccae	15483	1.2
40	M. xenopi	19971	1.5

SUBSTITUTE SHEET

As shown in Table 3 the probe also did not react with the rRNA from any of the respiratory pathogens which were also tested by the method just described. Nor did the probe
5 react with any other closely related or phylogenetically more diverse species of bacteria also tested by that method (Table 4).

TABLE 3

HYBRIDIZATION OF M. AVIUM PROBE TO
RESPIRATORY PATHOGENS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Corynebacterium xerosis	373	0.7
	Fusobacterium nucleatum	25586	1.3
	Haemophilum influenzae	19418	1.3
10	Klebsiella pneumoniae	23357	1.8
	Legionella pneumophila	33152	0.0
	Mycoplasma pneumoniae	15531	3.0
	Neisseria meningitidis	13090	0.0
	Pseudomonas aeruginosa	25330	0.0
15	Propionibacterium acnes	6919	1.1
	Streptococcus pneumoniae	6306	0.0
	Staphylococcus aureus	25923	1.5

TABLE 4

HYBRIDIZATION OF THE M. AVIUM PROBE TO A PHYLOGENETIC
CROSS SECTION OF BACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter calcoaceticus	33604	0.0
25	Branhamella catarrhalis	25238	0.6
	Bacillus subtilis	6051	0.9
	Bacteroides fragilis	23745	1.0
	Campylobacter jejuni	33560	0.4
	Chromobacterium Violaceum	29094	1.7
30	Clostridium perfringens	13124	2.1
	Deinococcus radiodurans	35073	0.8
	Derxia gummosa	15994	0.3
	Enterobacter aerogenes	13048	0.6
	Escherichia coli	11775	0.3
35	Mycobacterium gordonae	14470	1.9
	Mycoplasma hominis	14027	3.3
	Proteus mirabilis	29906	0.0
	Pseudomonas cepacia	11762	1.0
	Rahnella aquatilis	33071	2.1
40	Rhodospirillum rubrum	11170	0.6
	Streptococcus mitis	9811	0.9
	Vibrio parahaemolyticus	17802	1.2
	Yersinia enterocolitica	9610	0.4

SUBSTITUTE SHEET

Example 2

After the alignment described in Example 1, the following sequence was characterized by the
5 aforementioned criteria of length, T_m and sequence analysis and was determined to be specific for Mycobacterium intracellulare:

ACCGCAAAAGCTTTCCACCTAAAGACATGCGCCTAAAG

The sequence is complementary to a unique segment found
10 in the 16S rRNA of Mycobacterium intracellulare. The size of the probe was 38 bases. The probe has a T_m of 75°C and sequence analysis confirmed that the probe was correctly synthesized. The probe hybridizes to RNA of M. intracellulare in the region corresponding to bases
15 185-225 of E. coli 16S rRNA.

To demonstrate the reactivity of this sequence for the Mycobacterium intracellulare, the probe was tested in hybridization reactions under the following conditions. ^{32}P -end-labelled oligonucleotide probe was
20 mixed with 1 microgram (7×10^{-13} moles) of purified rRNA from Mycobacterium intracellulare and reacted in 0.12 M PB (equimolar amounts of Na_2HPO_4 and NaH_2PO_4), 1 mM EDTA and 0.2% SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final volume of 50 microliters. In
25 separate tubes the probe was mixed with the hybridization buffer with and without target Mycobacterium intracellulare rRNA present. Following separation on hydroxyapatite as outlined previously the hybrids were quantitated by scintillation counting.
30 These results are shown in Table 5.

SUBSTITUTE SHEET

TABLE 5

HYBRIDIZATION OF THE M. INTRACELLULARE
 PROBE TO HOMOLOGOUS TARGET rRNA*/

	<u>plus rRNA</u>	<u>minus rRNA</u>
<u>M. intracellulare</u> probe	85-95%	0.5%
* % Hybridization = $\frac{\text{cpm bound to hydroxyapatite}}{\text{total cpm added to reaction}}$		

10

These data shows that the probe has a high extent of reaction to its homologous target and very little non-specific binding to the hydroxyapatite.

Specificity of the Mycobacterium intracellulare probe was tested by mixing the ³²P labelled probe with rRNA released from cells from 29 other species of mycobacteria by sonic disruption techniques described in Murphy et. al. U.S. Patent Application No. 841,860. All hybridization assays were carried out as described in Example 1. Table 6 indicates that the probe is specific for Mycobacterium intracellulare and does not react with any other mycobacterial species, including Mycobacterium avium. These results are impressive in view of the 98% rRNA homology to M. avium; 98% homology to M. kansasii; 98% homology to M. asiaticum; and 97% homology to M. tuberculosis.

SUBSTITUTE SHEET

TABLE 6

HYBRIDIZATION OF THE M. INTRACELLULARE PROBE
TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	<i>Mycobacterium africanum</i>	25420	0.9
	<i>M. asiaticum</i>	25276	1.1
	<i>M. avium</i>	25291	1.3
10	<i>M. bovis</i>	19210	1.1
	<i>M. bovis</i> (BCG)	19015	1.2
	<i>M. chelonae</i>	14472	1.0
	<i>M. faveszens</i>	14474	1.2
	<i>M. fortuitum</i>	6841	1.3
15	<i>M. gastri</i>	15754	1.3
	<i>M. gordonae</i>	14470	1.3
	<i>M. haemophilum</i>	29548	0.9
	<i>M. intracellulare</i>	13950	78.8
	<i>M. kansasii</i>	12479	1.1
20	<i>M. Malmoense</i>	29571	1.0
	<i>M. marinum</i>	827	0.9
	<i>M. nonchromogenicum</i>	1930	1.0
	<i>M. phlei</i>	11758	1.1
	<i>M. scrofulaceum</i>	19981	1.0
25	<i>M. shimoidei</i>	27962	1.3
	<i>M. simiae</i>	25275	1.1
	<i>M. smegmatis</i>	e14468	1.3
	<i>M. szulgai</i>	23069	1.0
	<i>M. terrae</i>	15755	1.4
30	<i>M. thermoresistibile</i>	19527	1.6
	<i>M. triviale</i>	23292	1.3
	<i>M. tuberculosis</i> (avirulent)	25177	1.2
	<i>M. tuberculosis</i> (virulent)	27294	1.2
	<i>M. ulcerans</i>	19423	1.1
35	<i>M. vaccae</i>	15483	1.0
	<i>M. xenopi</i>	19971	1.2

As shown in Table 7 the probe did not react with
the rRNA from any of the respiratory pathogens tested in
the hybridization assay. Nor did the probe react with
any other closely related or phylogenetically more
diverse species of bacteria that were tested (Table 8).

TABLE 7

SUBSTITUTE SHEET

HYBRIDIZATION OF THE M. INTRACELLULARE PROBE
TO RESPIRATORY PATHOGENS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Corynebacterium xerosis	373	2.2
5	Fusobacterium nucleatum	25586	1.5
	Haemophilum influenzae	19418	1.3
	Klebsiella pneumoniae	23357	1.2
	Legionella pneumophila	33152	1.2
	Mycoplasma pneumoniae	15531	3.2
10	Neisseria meningitidis	13090	1.1
	Pseudomonas aeruginosa	25330	1.0
	Propionibacterium acnes	6919	2.9
	Streptococcus pneumoniae	6306	1.6
	Staphylococcus aureus	25923	1.3

TABLE 8

HYBRIDIZATION OF THE M. INTRACELLULARE PROBE
TO A PHYLOGENETIC CROSS SECTION OF BACTERIAL SPECIES

	<u>Organism</u>	<u>ATTC#</u>	<u>% Probe</u>
20	Acinetobacter calcoaceticus	33604	1.5
	Branhamella catarrhalis	25238	1.8
	Bacillus subtilis	6051	1.7
	Bacteroides fragilis	23745	1.9
25	Campylobacter jejuni	33560	1.9
	Chromobacterium Violaceum	29094	1.4
	Clostridium perfringens	13124	2.1
	Deinococcus radiodurans	35073	2.1
	Derxia gummosa	15994	1.6
30	Enterobacter aerogenes	13048	1.3
	Escherichia coli	11775	1.2
	Mycobacterium gordonae	14470	2.3
	Mycoplasma hominis	14027	2.6
	Proteus mirabilis	29906	1.2
35	Pseudomonas cepacia	11762	1.7
	Rahnella aquatilis	33071	1.5
	Rhodospirillum rubrum	11170	1.4
	Strptococcus mitis	9811	1.4
	Vibrio parahaemolyticus	17802	2.5
40	Yersinia enterocolitica	9610	1.1

Example 3

SUBSTITUTE SHEET

After the alignment described in Example 1, the following sequence was characterized by the aforementioned three criteria of size, sequence and T_m, and was determined to be specific to the Mtb complex of organisms, Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium bovis, and Mycobacterium microti:

1. TAAAGCGCTTTCCACCACAAGACATGCATCCCGTG.

The sequence is complementary to a unique segment found in the 16S rRNA of the Mtb-complex bacteria. The size of the probe is 35 bases. The probe has a T_m of 72°C and sequence analysis confirmed that the probe was correctly synthesized. It is capable of hybridizing in the region corresponding to bases 185-225 of E. coli 16S rRNA.

To demonstrate the reactivity of this sequence for the Mtb complex the probe was tested in hybridization reactions under the following conditions. ³²P-end-labelled oligonucleotide probe was mixed with 1 microgram (7x10⁻¹³ moles) of purified rRNA from Mycobacterium tuberculosis and reacted in 0.12 M PB hybridization buffer (equimolar amounts of Na₂HPO₄, and NaH₂PO₄), 1 mM EDTA and 0.2 SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was mixed with the hybridization buffer with and without target rRNA from Mycobacterium tuberculosis present. Following separation on hydroxyapatite as outlined previously the hybrids were quantitated by scintillation counting. The results are shown in Table 9.

TABLE 9

HYBRIDIZATION OF Mtb-COMPLEX 16S rRNA DNA
PROBE TO HOMOLOGOUS TARGET rRNA*/

	<u>plus rRNA</u>	<u>minus rRNA</u>
Mtb complex probe	85-95%	0.5%
* % Hybridization = $\frac{\text{cpm bound to hydroxyapatite}}{\text{total cpm added to reaction}}$		

This data shows that the probe has a high extent of reaction to homologous target and very little non-specific binding to the hydroxyapatite.

Specificity of the probe for the Mtb complex was tested by mixing the ³²P labelled probe with rRNA released from cells of the 4 Mtb complex bacilli and of 25 other mycobacterial species by sonic disruption techniques described in Murphy et. al., U.S. Patent Application No. 841,860. All hybridization assays were carried out as described in Example 1. Table 10 indicates that the probe is specific for organisms within the Mtb complex and does not react with any other mycobacterial species.

TABLE 10

HYBRIDIZATION OF Mtb-COMPLEX 16S rRNA DNA
PROBE TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Mycobacterium africanum	25420	68.1
	M. asiaticum	25276	3.4
35	M. avium	25291	0.9
	M. bovis	19210	63.1

	M. chelonae	14472	1.1
	M. flavescens	14474	0.9
	M. fortuitum	6841	1.1
	M. gastri	15754	0.8
5	M. gordonae	14470	1.1
	M. haemophilum	29548	0.8
	M. intracellulare	13950	1.1
	M. kansasii	12479	1.3
	M. malmoense	29571	0.9
10	M. marinum	827	1.1
	M. nonchromogenicum	1930	1.1
	M. phlei	11758	1.3
	M. scrofulaceum	19981	1.1
	M. shimoidei	27962	1.0
15	M. simiae	25275	1.2
	M. smegmatis	el4468	0.9
	M. szulgai	23069	1.1
	M. terrae	15755	1.0
	M. thermoresistibile	19527	1.0
20	M. triviale	23292	1.2
	M. tuberculosis (avirulent)	25177	66.2
	M. tuberculosis (virulent)	27294	62.4
	M. ulcerans	19423	0.9
	M. vaccae	15483	0.8
25	M. xenopi	19971	2.6

As shown in Table 11 the probe did not react with the rRNA from any of the respiratory pathogens tested in the hybridization assay. Nor did the probe react with any other closely related or phylogenetically more diverse species of bacteria that were tested (Table 12).

35

TABLE 11

HYBRIDIZATION OF Mtb-COMPLEX 16S rRNA DNA
PROBE TO RESPIRATORY PATHOGENS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
40	Corynebacterium xerosis	373	1.3
	Fusobacterium nucleatum	25586	1.0
	Haemophilum influenzae	19418	1.6
	Klebsiella pneumoniae	23357	1.2
	Legionella pneumophila	33152	1.4
45	Mycoplasma pneumoniae	15531	1.1
	Neisseria meningitidis	13090	1.0

Pseudomonas aeruginosa	25330	1.7
Propionibacterium acnes	6919	1.2
Streptococcus pneumoniae	25923	0.9

5

TABLE 12

HYBRIDIZATION OF THE Mtb-COMPLEX 16S rRNA
DNA PROBE TO A PHYLOGENETIC CROSS SECTION
OF BACTERIAL SPECIES

10	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe</u>
	Acinetobacter calcoaceticus	33604	1.3
	Branhamella catarrhalis	25238	1.5
	Bacillus subtilis	6051	1.3
	Bacteroides fragilis	23745	1.3
15	Campylobacter jejuni	33560	1.1
	Chromobacterium violaceum	29094	1.0
	Clostridium perfringens	13124	1.2
	Deinococcus radiodurans	35073	1.0
	Derxia gummosa	15994	1.0
20	Enterobacter aerogenes	13048	1.0
	Escherichia coli	11775	1.0
	Mycobacterium gordonae	14470	1.3
	Mycoplasma hominis	14027	0.5
	Proteus mirabilis	29906	1.0
25	Pseudomonas cepacia	11762	2.6
	Rahnella aquatilis	33071	1.9
	Rhodospirillum rubrum	11170	1.0
	Streptococcus mitis	9811	1.1
	Vibrio parahaemolyticus	17802	0.9
30	Yersinia enterocolitica	9610	1.1

Two derivatives of the probe of Example 3
(numbered 2-3 below) were made and tested:

- 35 2. CCGCTAAAGCGCTTTCCACCACAAGACATGCATCCCG
 3. ACACCGCTAAAGCGCTTTCCACCACAAGACATGCATC.

All three probes have similar Tms (72°; 73.5°; and
72.3°, respectively) and similar hybridization
characteristics.

- 40 Hybridization to Mycobacterium tuberculosis
complex organisms was 68-75% and non-specific
hybridization to hydroxyapatite was less than 2%.

SUBSTITUTE SHEET

Results of hybridization assay tests for these derivatives follow.

5

TABLE 13

HYBRIDIZATION OF PROBE OF EXAMPLES 3 AND 2
DERIVATIVES THEREOF
TO MYCOBACTERIAL SPECIES

10

	Organism	ATCC#	Example	%	%	%
			Probe 1	Probe 2	Probe 3	
			Bound	Bound	Bound	
	Mycobacterium	25420	68.1	69.4	70.6	
15	africanum					
	M. asiaticum	25274	3.4	5.3	1.8	
	M. avium	25291	.9	1.6	1.4	
	M. bovis	19210	63.1	75.3	74	
	M. chelonae	14472	1.1	1.5	1.6	
20	M. flavescens	14474	0.9	2.7	1.4	
	M. fortuitum	6841	1.1	3.6	1.5	
	M. gastri	15754	0.8	3.6	1.7	
	M. gordonae	14470	1.1	1.6	1.4	
	M. haemophilum	29548	0.8	3.2	1.7	
25	M. intracellulare	13950	1.1	1.6	1.4	
	M. kansasii	12478	1.3	2.1	2.0	
	M. malmoense	29571	0.9	2.8	1.5	
	M. marinum	827	1.1	2.1	1.5	
	M. nonchromogenicum	1930	1.1	3.0	1.5	
30	M. phlei	11758	1.3	1.3	1.1	
	M. scrofulaceum	19981	1.1	3.4	1.6	
	M. shimoidi	27962	1.0	2.7	1.6	
	M. simiae	25275	1.2	2.9	1.8	
	M. smegmatis	14468	0.9	1.5	1.2	
35	M. szulgai	23069	1.1	3.6	1.1	
	M. terrae	15755	1.0	3.7	2.0	
	M. thermo-					
	resistibile	19527	1.0	1.6	1.3	
	M. triviale	23292	1.2	1.6	2.0	
40	M. tuberculosis					
	(avirulent)	25177	66.2	75	68	
	M. tuberculosis					
	(virulent)	27294	62.4	74	75	
	M. ulcerans	19423	0.9	1.7	3.0	
45	M. vaccae	15483	0.8	1.4	1.2	
	M. xenopi	19971	2.6	1.4	1.2	

SUBSTITUTE SHEET

Example 4

The probe specific for the 23S rRNA of the M. tuberculosis complex was obtained by using a primer which was complementary to a highly conserved region of 23S rRNA. The sequence of this primer, derived from E. coli rRNA, was 5'-AGG AAC CCT TGG GCT TTC GG-3'. Five nanograms of this primer was mixed with 1 microgram of rRNA from M. tuberculosis and other closely related Mycobacterium and the procedure as described for Examples 1, 2 and 3 was followed. After alignment as described in Example 1, the following sequence was determined to be specific to the Mtb complex of organisms, Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium bovis, and Mycobacterium microti:

TGCCCTACCCACACCCACCACAAGGTGATGT.

The sequence is complementary to a unique segment found in the 23S rRNA of the Mtb-complex bacteria. The oligonucleotide probe was characterized as previously described by the criteria of length, T_m and sequence analysis. The size of the probe is 31 bases. The probe has a T_m of 72.5°C and sequence analysis confirmed that the probe was correctly synthesized. It is capable of hybridizing in the region corresponding to bases 1155-1190 of E. coli 23S rRNA.

To demonstrate the reactivity of this sequence for the Mtb complex the probe was tested in hybridization reactions under the following conditions. ^{32}P -end-labelled oligonucleotide probes were mixed with 1 microgram (7×10^{-13} moles) of purified rRNA from Mycobacterium tuberculosis and reacted in 0.12 M PB hybridization buffer (equimolar amounts of Na_2HPO_4 , and NaH_2PO_4), 1 mM EDTA and 0.2 SDS (sodium dodecyl sulfate)

SUBSTITUTE SHEET

at 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was mixed with the hybridization buffer with and without target rRNA from Mycobacterium tuberculosis present. Following separation on hydroxyapatite as outlined previously the hybrids were quantitated by scintillation counting. The results are shown in Table 14.

10

TABLE 14

HYBRIDIZATION OF THE Mtb-COMPLEX
23S rRNA DNA PROBE TO HOMOLOGOUS TARGET rRNA

		<u>plus rRNA</u>	<u>minus rRNA</u>
15	Mtb complex 23S probe	94%	1.2%

These data show that the probe has a high extent of reaction to homologous target and very little non-specific binding to the hydroxyapatite.

Specificity of the probe for the Mtb complex was tested by mixing the ³²P labelled probe with rRNA released from cells of the four Mtb complex bacilli and of 25 other mycobacterial species by sonic disruption techniques described in Murphy et al., U.S. Patent Application No. 841,860. All hybridization assays were carried out as described in Example 1. Table 14 indicates that the probe is specific for organisms within the Mtb complex and does not react with any other mycobacterial species.

SUBSTITUTE SHEET

TABLE 15

5 HYBRIDIZATION OF Mtb-COMPLEX 23S rRNA DNA PROBE
TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Mycobacterium africanum	25420	33.6
	M. asiaticum	25276	1.2
10	M. avium	25291	1.0
	M. bovis	19210	32.0
	M. chelonae	14472	1.2
	M. flavescens	14474	1.2
	M. fortuitum	6841	1.3
15	M. gastri	15754	1.1
	M. gordonae	14470	1.2
	M. haemophilum	29548	1.2
	M. intracellulare	13950	1.1
	M. kansasii	12479	1.3
20	M. malmoense	29571	1.3
	M. marinum	827	1.2
	M. nonchromogenicum	1930	1.0
	M. phlei	11758	1.0
	M. scrofulaceum	19981	1.1
25	M. shimoides	27962	1.2
	M. simiae	25275	1.3
	M. smegmatis	el4468	1.1
	M. szulgai	23069	1.1
	M. terrae	15755	1.0
30	M. thermoresistibile	19527	1.2
	M. triviale	23292	1.0
	M. tuberculosis (avirulent)	25177	33.7
	M. tuberculosis (virulent)	27294	38.1
	M. ulcerans	19423	1.3
35	M. vaccae	15483	1.0
	M. xenopi	19971	1.3

Example 5

40 Three additional Mycobacterium tuberculosis complex probes, Examples 5-7 herein, were identified using two unique primers complementary to 23S rRNA. The first sequence is:

CCATCACCACCCTCCTCCGGAGAGGAAAAGG.

The sequence of this Example 5 was obtained using a 23S primer with the sequence 5'-GGC CAT TAG ATC ACT CC-3'. It was characterized and shown to be specific for the Mycobacterium tuberculosis complex of organisms including Mycobacterium tuberculosis, Mycobacterium africanum and Mycobacterium bovis. This sequence, from 23S rRNA, is 31 bases in length and has a T_m of 72°C. This probe is capable of hybridizing to RNA of the
10 aforementioned organisms in the region corresponding to bases 540-575 of E. coli 23S rRNA.

To demonstrate the reactivity and specificity of this probe for Mycobacterium tuberculosis complex, it was tested as a probe in hybridization reactions under
15 the following conditions. ³²P-end-labeled oligonucleotide probe was mixed with rRNA released from cells of 30 species of mycobacteria by the sonic disruption techniques described in Murphy et al., U.S. Patent Application Serial No. 841,860. 3 x 10⁷ cells
20 were suspended in 0.1 ml 5% SDS and sonicated for 15 minutes at 50-60°C. One ml of hybridization buffer (45% diisobutyl sulfosuccinate, 40 mM phosphate buffer pH 6.8, 1 mM EDTA, 1 mM EGTA) was added and the mixture incubated at 72°C for 2 hours. Following incubation, 4
25 ml of 2% (w/v) hydroxyapatite, 0.12M sodium phosphate buffer pH6.8, 0.02% SDS, 0.02% sodium azide was added and incubated at 72°C for 5 minutes. The sample was centrifuged and the supernatant removed. Four ml wash solution (0.12M sodium phosphate buffer pH6.8, 0.02%
30 SDS, 0.02% sodium azide) was added and the sample was vortexed, centrifuged and the supernatant removed. The radioactivity bound to the hydroxyapatite was determined by scintillation counting. The results are shown in Table 16 and indicate that the probe is

specific for the Mycobacterium tuberculosis complex of organisms.

5

TABLE 16

HYBRIDIZATION OF THE M. TUBERCULOSIS COMPLEX
PROBE OF EXAMPLE 5 TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC #</u>	<u>% Probe Bound</u>
10	<i>Mycobacterium africanum</i>	25420	18.0
	<i>M. asiaticum</i>	25274	2.6
	<i>M. avium</i>	25291	3.4
	<i>M. bovis</i>	19210	21.7
	<i>M. bovis</i> (BCG)	35734	35.3
15	<i>M. chelonae</i>	14472	3.8
	<i>M. flavescens</i>	14474	2.3
	<i>M. fortuitum</i>	6841	1.8
	<i>M. gastri</i>	15754	2.2
	<i>M. gordonae</i>	14470	2.8
20	<i>M. haemophilum</i>	29548	2.8
	<i>M. intracellulare</i>	13950	2.1
	<i>M. kansasii</i>	12478	1.6
	<i>M. malmoense</i>	29571	2.3
	<i>M. marinum</i>	827	2.1
25	<i>M. nonchromogenicum</i>	1930	2.3
	<i>M. phlei</i>	11758	2.1
	<i>M. scrofulaceum</i>	19981	2.2
	<i>M. shimoidei</i>	27962	1.9
	<i>M. simiae</i>	25275	2.2
30	<i>M. smegmatis</i>	el4468	2.0
	<i>M. szulgai</i>	23069	2.2
	<i>M. terrae</i>	15755	2.2
	<i>M. thermoresistibile</i>	19527	2.2
	<i>M. triviale</i>	23292	2.0
35	<i>M. tuberculosis</i> (avirulent)	25177	26.4
	<i>M. tuberculosis</i> (virulent)	27294	36.6
	<i>M. ulcerans</i>	19423	2.5
	<i>M. vaccae</i>	15483	2.4
40	<i>M. xenopi</i>	19971	2.8

Table 16 shows that the probe also did not cross react with RNA from any of the closely related organisms tested by the method just described.

TABLE 17

5 HYBRIDIZATION OF THE M. TUBERCULOSIS
 COMPLEX PROBE OF EXAMPLE 5 TO PHYLOGENETICALLY
 CLOSELY RELATED ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Actinomadura madurae	19425	2.1
10	Actinoplanes italicus	10049	3.1
	Arthrobacter oxidans	14358	2.1
	Brevibacterium linens	e9172	1.9
	Corynebacterium xerosis	373	2.2
	Dermatophilus congolensis	14367	2.2
15	Microbacterium lacticum	8180	2.1
	Nocardia asteroides	19247	2.0
	Nocardia brasiliensis	19296	2.2
	Nocardia otitidis-caviarum	14629	2.0
	Nocardiopsis dassonvillei	23218	4.0
20	Oerskovia turbata	33225	2.2
	Oerskovia xanthineolytica	27402	2.0
	Rhodococcus aichiensis	33611	1.9
	Rhodococcus aurantiacus	25938	2.0
	Rhodococcus bronchialis	25592	2.1
25	Rhodococcus chubuensis	33609	2.3
	Rhodococcus equi	6939	2.4
	Rhodococcus obuensis	33610	2.2
	Rhodococcus sputi	29627	2.3
30			

Example 6

The second Mycobacterium tuberculosis complex probe was obtained using a 23S primer with the sequence 5' CCT GAT TGC CGT CCA GGT TGA GGG AAC CTT TGG G-3'.

35 Its sequence is:

CTGTCCCTAAACCCGATTCAGGGTTCGAGGTTAGATGC

This sequence, from 23S rRNA, is 38 bases in length and has a T_m of 75°C. It hybridizes in the region corresponding to bases 2195-2235 of E. coli 23s rRNA.

40 Like the complex probe in Example 5, this sequence was characterized and shown to be specific for the Mycobacterium tuberculosis complex of organisms

SUBSTITUTE SHEET

including Mycobacterium tuberculosis, Mycobacterium africanum and Mycobacterium bovis.

To demonstrate the reactivity and specificity of the probe of this Example 6 to Mycobacterium tuberculosis complex, it was tested as a probe in hybridization reactions under the following conditions described for the probe in Example 5. The results are shown in Table 18 and indicate that the probe is specific for the Mycobacterium tuberculosis complex of organisms with the exception of Mycobacterium thermoresistibile, a rare isolate which is not a human pathogen.

15

TABLE 18

HYBRIDIZATION OF THE M. TUBERCULOSIS COMPLEX
PROBE OF EXAMPLE 6 TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC #</u>	<u>% Probe Bound</u>
20	<u>Mycobacterium africanum</u>	25420	56.0
	M. asiaticum	25274	3.1
	M. avium	25291	2.6
	M. bovis	19210	48.0
	M. bovis (BCG)	35734	63.0
25	M. chelonae	14472	2.8
	M. flavescens	14474	2.8
	M. fortuitum	6841	3.0
	M. gastri	15754	3.2
	M. gordonae	14470	3.0
30	M. haemophilum	29548	3.0
	M. intracellulare	13950	3.6
	M. kansasii	12478	3.9
	M. malmoense	29571	2.9
	M. marinum	827	2.9
35	M. nonchromogenicum	1930	4.8
	M. phlei	11758	2.9
	M. scrofulaceum	19981	2.6
	M. shimoidi	27962	3.6
	M. simiae	25275	3.3
40	M. smegmatis	el4468	3.0
	M. szulgai	23069	2.8
	M. terrae	15755	2.8

	M. thermoresistibile	19527	11.7
	M. triviale	23292	3.2
	M. tuberculosis (avirulent)	25177	65.0
	M. tuberculosis (virulent)	27294	53.0
5	M. ulcerans	19423	2.5
	M. vaccae	15483	2.8
	M. xenopi	19971	3.3

10 Table 19 shows that the probe also did not cross react with RNA from any of the phylogenetically closely related organisms tested by the method just described.

15

TABLE 19

20 HYBRIDIZATION OF THE M. TUBERCULOSIS COMPLEX
PROBE OF EXAMPLE 6 TO PHYLOGENETICALLY
CLOSELY RELATED ORGANISMS

	<u>Organism</u>	<u>ATCC #</u>	<u>% Probe Bound</u>
	Actinomadura madurae	19425	1.3
	Actinoplanes italicus	10049	0.6
25	Arthrobacter oxidans	14358	1.1
	Brevibacterium linens	e9172	0.8
	Corynebacterium xerosis	373	1.0
	Dermatophilus congolensis	14367	0.6
	Microbacterium lacticum	8180	1.9
30	Nocardia asteroides	19247	0.9
	Nocardia brasiliensis	19296	0.8
	Nocardia otitidis-caviarum	14629	1.5
	Nocardioopsis dassonvillei	23218	0.5
	Oerskovia turbata	33225	0.3
35	Oerskovia xanthineolytica	27402	0.8
	Rhodococcus aichiensis	33611	1.6
	Rhodococcus aurantiacus	25938	0.7
	Rhodococcus bronchialis	25592	1.5
	Rhodococcus chubuensis	33609	0.8
40	Rhodococcus equi	6939	0.3
	Rhodococcus obuensis	33610	0.8
	Rhodococcus sputi	29627	1.4

45

SUBSTITUTE SHEET

Example 7

The following additional Mycobacterium tuberculosis complex probe also has been identified using a 23S primer with

5 the same sequence as that of Example 6, namely, 5'-CCT
GAT TGC CGT CCA GGT TGA GGG AAC CTT TGG G-3':

AGGCACTGTCCCTAAACCCGATTCAGGGTTC.

This sequence, from 23S rRNA is 31 bases in length and has a T_m of 71°C. It hybridizes in the region
10 corresponding to bases 2195-2235 of E. coli 23S rRNA. As is the case with the Mycobacterium tuberculosis complex probes of Examples 5 and 6 herein, this sequence also was characterized and shown to be specific for the
15 Mycobacterium tuberculosis complex of organisms, including Mycobacterium tuberculosis, Mycobacterium africanum and Mycobacterium bovis.

To demonstrate the reactivity and specificity of this probe for Mycobacterium tuberculosis complex, it was tested as a probe in hybridization reactions under
20 the conditions described for the probe of Example 5. Table 20 shows that the probe is specific for the Mycobacterium tuberculosis complex of organisms.

25

TABLE 20

HYBRIDIZATION OF THE MYCOBACTERIUM
TUBERCULOSIS COMPLEX PROBE OF EXAMPLE 7
TO MYCOBACTERIAL SPECIES

30	<u>Organism</u>	<u>ATCC #</u>	<u>% Probe Bound</u>
	<u>Mycobacterium africanum</u>	25420	43.0
	<u>M. asiaticum</u>	25274	0.6
	<u>M. avium</u>	25291	0.7
	<u>M. bovis</u>	19210	43.0
35	<u>M. bovis (BCG)</u>	35734	46.0
	<u>M. chelonae</u>	14472	0.6
	<u>M. flavescens</u>	14474	0.6
	<u>M. fortuitum</u>	6841	0.5

SUBSTITUTE SHEET

	M. gastri	15754	0.9
	M. gordonae	14470	0.7
	M. haemophilum	29548	0.6
	M. intracellulare	13950	0.6
5	M. kansasii	12478	0.9
	M. malmoense	29571	0.8
	M. marinum	827	0.7
	M. nonchromogenicum	1930	0.8
	M. phlei	11758	0.6
10	M. scrofulaceum	19981	0.7
	M. shimoidi	27962	0.8
	M. simiae	25275	0.7
	M. smegmatis	el4468	0.6
	M. szulgai	23069	0.6
15	M. terrae	15755	0.7
	M. thermoresistibile	19527	0.9
	M. triviale	23292	0.7
	M. tuberculosis (avirulent)	25177	40.0
	M. tuberculosis (virulent)	27294	50.0
20	M. ulcerans	19423	0.7
	M. vaccae	15483	0.4
	M. xenopi	19971	0.6

25 Table 21 shows that the probe also did not cross react with RNA from any of the closely related organisms tested by the method just described.

30

TABLE 21

HYBRIDIZATION OF THE M. TUBERCULOSIS COMPLEX
PROBE OF EXAMPLE 7 TO PHYLOGENETICALLY
CLOSELY RELATED ORGANISMS

35	<u>Organism</u>	<u>ATCC #</u>	<u>% Probe Bound</u>
	Actinomadura madurae	19425	1.0
	Actinoplanes italicus	10049	0.6
	Arthrobacter oxidans	14358	0.4
	Brevibacterium linens	e9172	0.8
40	Corynebacterium xerosis	373	0.6
	Dermatophilus congolensis	14367	0.8
	Microbacterium lacticum	8180	0.5
	Nocardia asteroides	19247	0.7
	Nocardia brasiliensis	19296	0.5
45	Nocardia otitidis-caviarum	14629	0.6
	Nocardiopepsis dassonvillei	23218	0.6
	Oerskovia turbata	33225	0.8

SUBSTITUTE SHEET

	Oerskovia xanthineolytica	27402	0.6
	Rhodococcus aichiensis	33611	0.7
	Rhodococcus aurantiacus	25938	0.7
	Rhodococcus bronchialis	25592	0.6
5	Rhodococcus chubuensis	33609	0.6
	Rhodococcus equi	6939	0.6
	Rhodococcus obuensis	33610	0.6
	Rhodococcus sputi	29627	0.9

10

Notably, overlapping probes may have identical specificity. Compare, for example, the probes of Examples 6 and 7:

Ex. 6 CTGTCCCTAAACCCGATTCAGGGTTCGAGGTTAGATGC
 15 Ex. 7 AGGCACTGTCCCTAAACCCGATTCAGGGTTC

There may be several sequences from a particular region which will yield probes with the desired hybridization characteristics. In other cases, one probe sequence may be significantly better than another probe differing by a single
 20 base. In general, the greater the sequence difference (% mismatch) between a target and nontarget organism, the more likely one will be able to alter the probe without affecting its usefulness for a specific application. This phenomenon also was demonstrated by
 25 the derivative probes in Example 3.

In Example 7, five bases were added to the 5' end of the probe in Example 6, and 12 bases were removed from the 3' end. The two probes have essentially
 30 identical hybridization characteristics.

Example 8

The Mycobacterium genus is particularly difficult to distinguish from Nocardia, Corynebacterium and Rhodococcus. These genera have common antigens,
 35 precipitins and G & C counts. Despite the fact that these organisms also exhibit 92-94% rRNA homology to the

SUBSTITUTE SHEET

above listed Mycobacterium organisms, we have designed probes which detect all members of the genus Mycobacterium without cross reacting to the related genera.

5 In addition to the Mycobacterium species probes already disclosed, four probes specific for members of the Mycobacterium genus were identified using one primer complementary to 16S rRNA and one primer complementary to 23S rRNA. Sequence 1 was obtained using a 16S primer
10 with the sequence 5'-TTA CTA GCG ATT CCG ACT TCA-3'. Sequences 2, 3 and 4 were obtained using a 23S primer with the sequence 5'-GTG TCG GTT TTG GGT ACG-3'. Sequence 1 is capable of hybridizing to RNA of the genus Mycobacterium in the region corresponding to bases 1025-
15 1060 of E. coli 16S rRNA. Sequences 2-4 hybridize in regions corresponding to the following bases of E. coli 23S rRNA in our numbering system (See Figure 2); 1440-1475; 1515-1555; 1570-1610 in our numbering system.

The following sequences were characterized and
20 shown to be specific for the genus Mycobacterium:

1. CCA TGC ACC ACC TGC ACA CAG GCC ACA AGG
2. GGC TTG CCC CAG TAT TAC CAC TGA CTG GTA CGG
3. CAC CGA ATT CGC CTC AAC CGG CTA TGC GTC ACC TC
4. GGG GTA CGG CCC GTG TGT GTG CTC GCT AGA GGC

25 Sequence 1, from 16S rRNA, is 30 bases in length and has a T_m of 73°. Sequence 2, from 23S rRNA, is 33 bases in length and has a T_m of 75°C. Sequence 3, from 23S rRNA, is 35 bases in length and has a T_m of 76°C. Sequence 4, from 23S rRNA, is 33 bases in length and has
30 a T_m of 73°C.

To demonstrate the reactivity and specificity of probe 1 for members of the genus Mycobacterium, it was tested as a probe in hybridization reactions under the following conditions. ¹²⁵I-labeled oligonucleotide
35 probe was mixed with rRNA released from cells of 30

species of mycobacteria by the sonic disruption techniques described in Murphy et al., U.S. Patent Application Serial No. 841,860. 3×10^7 cells were suspended in 0.1 ml 5% SDS and sonicated for 15 minutes at 50-60°C. One ml of hybridization buffer (45% diisobutyl sulfosuccinate, 40 mM sodium phosphate pH6.8, 1 mM EDTA, 1 mM EGTA) was added and the mixture incubated at 72°C for 2 hours. Following incubation, 2 ml of separation solution (containing 2.5 g/l cationic magnetic microspheres, 0.17M sodium phosphate buffer pH6.8, 7.5% Triton X-100 (TM), 0.02% sodium azide) was added and incubated at 72°C for 5 minutes. The RNA:probe hybrids, bound to the magnetic particles, were collected and the supernatant removed. One ml wash solution (0.12M sodium phosphate buffer pH6.8, 14% diisobutyl sulfosuccinate, 5% Triton X-100, 0.02% sodium azide) was added, the particles collected and the supernatant removed. This step was repeated two times. The radioactivity bound to the magnetic particles was determined in a gamma counter. The results are shown in Table 22 and indicate that the probes hybridize to organisms in the genus Mycobacterium and that a combination of probes will detect all members of the genus. Table 23 shows that the probes do not react with other closely related bacteria.

TABLE 22

HYBRIDIZATION OF THE MYCOBACTERIUM PROBES 1-4 TO MYCOBACTERIAL SPECIES					
<u>Organism</u>	<u>ATCC#</u>	<u>% Probe 1 Bound</u>	<u>% Probe 2 Bound</u>	<u>% Probe 3 Bound</u>	<u>% Probe 4 Bound</u>
Mycobacterium	25420	41.5	14.7	17.9	26.7
35 africanum					
M. asiaticum	25274	31.8	20.2	7.9	0.1

	<i>M. avium</i>	25291	11.7	34.7	10.1	1.6
	<i>M. bovis</i>	19210	19.4	28.4	44.6	20.9
	<i>M. bovis</i> (BCG)	35734	30.0	35.5	17.8	5.6
5	<i>M. chelonae</i>	14472	8.6	0.7	6.3	0.2
	<i>M. flavescens</i>	14474	29.8	17.7	2.3	0.9
	<i>M. fortuitum</i>	6841	34.7	2.2	4.8	0.2
	<i>M. gastri</i>	15754	27.6	65.1	9.6	22.3
	<i>M. goodii</i>	14470	50.7	55.2	3.1	0.4
10	<i>M. haemo-</i> <i>philum</i>	29548	40.7	60.7	0.4	12.4
	<i>M. intracel-</i> <i>lulare</i>	3950	38.8	48.3	0.9	5.4
	<i>M. kansasii</i>	12478	53.4	27.3	24.5	27.8
15	<i>M. malmoense</i>	29571	3.1	38.4	0.8	1.5
	<i>M. marinum</i>	827	41.7	4.1	4.8	0.1
	<i>M. nonchro-</i> <i>mogenicum</i>	1930	35.0	42.9	0.5	16.4
	<i>M. phlei</i>	11758	23.7	0.6	1.8	0.6
20	<i>M. scroful-</i> <i>aceum</i>	19981	35.1	66.9	0.9	26.4
	<i>M. shimoides</i>	27962	34.6	1.4	1.3	4.8
	<i>M. simiae</i>	25275	45.9	44.0	5.3	0.1
	<i>M. smegmatis</i>	14468	31.3	4.0	5.6	0.1
25	<i>M. szulgai</i>	23069	19.4	22.3	1.5	3.0
	<i>M. terrae</i>	15755	25.6	21.7	0.4	12.3
	<i>M. thermore-</i> <i>sistibile</i>	19527	20.3	34.5	3.1	17.6
	<i>M. triviale</i>	23292	37.3	4.6	4.3	0.1
30	<i>M. tuberculo-</i> <i>sis (avir)</i>	25177	38.5	26.3	11.3	23.0
	<i>M. tuberculo-</i> <i>sis (virul)</i>	27294	13.8	12.4	38.4	22.3
	<i>M. ulcerans</i>	19423	33.9	28.7	0.4	8.9
35	<i>M. vaccae</i>	15483	8.8	36.2	4.8	3.2
	<i>M. xenopi</i>	19971	38.4	2.1	3.8	0.2

40

TABLE 23

HYBRIDIZATION OF THE MYCOBACTERIUM PROBES
1-4 TO PHYLOGENETICALLY CLOSELY RELATED ORGANISMS

		#	% Probe	% Probe	% Probe	% Probe
	<u>Organism</u>	<u>ATCC</u>	<u>1 Bound</u>	<u>2 Bound</u>	<u>3 Bound</u>	<u>4 Bound</u>
45	Actinomad- ura madurae	19425	0.2	0.3	0.2	0.1
	Actinoplanes	10049	0.4	0.5	0.3	0.2

SUBSTITUTE SHEET

	<i>italicus</i>				
	Arthrobacter 14358	0.2	0.4	0.3	0.1
	oxidans				
	Brevibact- e9172	0.3	0.3	0.3	0.1
5	erium linens				
	Corynebact- 373	0.4	0.3	0.3	0.1
	erium xerosis				
	Dermatoph- 14367	0.4	0.6	0.3	0.2
	ilus congolensis				
10	Microbact- 8180	0.2	0.3	0.2	0.1
	erium lacticum				
	Nocardia 19247	0.3	0.3	0.4	0.1
	asteroides				
	Nocardia 19296	0.4	0.3	0.6	0.1
15	brasili-				
	ensis				
	Nocardia 14629	0.4	0.4	1.0	0.3
	otitidis-				
	caviarum				
20	Nocardio-	0.3	0.2	0.3	0.1
	osis				
	dassonvillei				
	Oerskovia 33225	0.2	0.2	0.3	0.1
	turbata				
25	Oerskovia 27402	0.2	0.3	0.3	0.1
	xanthineolytica				
	Rhodococcus 33611	0.4	0.2	0.3	0.2
	aichiensis				
	Rhodococcus 25938	0.3	0.4	0.3	0.2
30	aurantiacus				
	Rhodococcus 25592	0.4	0.3	0.3	0.1
	bronchialis				
	Rhodococcus 33609	0.6	0.4	0.3	0.3
	chubuensis				
35	Rhodococcus 6939	0.4	0.4	0.4	0.5
	equi				
	Rhodococcus 33610	0.5	0.5	0.3	0.1
	obuensis				
	Rhodococcus 29627	0.4	0.5	0.4	0.3
40	sputi				

Example 9

45 Mycoplasmas are small, aerobic bacteria lacking cell walls. Mycoplasma pneumoniae is estimated to cause 8-15 million infections per year. The infections may be asymptomatic or range in severity from mild to severe bronchitis and pneumonia. The organism is believed to

SUBSTITUTE SHEET

cause about 10% of pneumonias in the general population and 10-50% of the pneumonias of members of groups in prolonged, close contact such as college students and military personnel.

5 Diagnosis until now has required isolation of the organism in culture or demonstration of an increase in antibody titer. Culturing of the organism involves inoculation of respiratory tract specimens onto agar or biphasic media containing bacterial growth inhibitors.
10 Examination for growth at 3-4 and 7-10 days is used to establish the presence or absence of any mycoplasma. Mycoplasma pneumoniae must then be identified by hemadsorption (the ability of M. pneumoniae to adhere sheep or guinea pig erythrocytes), hemolysis (the
15 ability of M. pneumoniae to produce beta hemolysis of sheep or guinea pig erythrocytes in blood agar), growth inhibition by specific antibodies, or immunofluorescence with specific antibodies. The present invention has significant advantages over each
20 of these prior art methods both because of the simplicity of the test and because of the greatly reduced time necessary to achieve a diagnosis.

 A probe specific for the 5S rRNA of M. pneumoniae was obtained by a comparison of known rRNA sequences.
25 The particular sequences aligned were from M. pneumoniae, M. gallisepticum and Ureaplasma urealyticum (Rogers, M.J. et al. 1985, Proc. Natl. Acad. Sci. USA, 82 (1160-1164), M. capricolum (Hori, H. et al. 1981, Nucl. Acids Res. 9, 5407-5410) and Spiroplasma sp.
30 (Walker, R.T. et al. 1982 Nucl. Acids Res. 10, 6363-6367). The alignments were performed as described above and outlined at page 6. 5S rRNA can be isolated and sequenced as outlined in Rogers et al., or a primer can be made which is complementary to a conserved region
35 in the 5S rRNA and sequencing performed as outlined in

SUBSTITUTE SHEET

Examples 1-4. The conserved region of 5S rRNA is documented in Fox, G.E. and Woese, C.R., 1975, Nature 256: 505-507. The following sequence was determined to be specific for *Mycoplasma pneumoniae*:

5 GCTTGGTGCTTTCCTATTCTCACTGAAACAGCTACATTCGGC.

The sequence is complementary to a unique segment found in the 5S rRNA of *Mycoplasma pneumoniae* in the region corresponding to bases 65-108 of *E. coli* 5S rRNA, and was selected by comparison to 5S rRNA sequences from *Mycoplasma gallisepticum*, *Spiroplasma mirum* and *Ureaplasma urealyticum*. The oligonucleotide probe was characterized as described above. The size of the probe was 42 bases. The probe has a T_m of 71.5°C.

To demonstrate the reactivity of this sequence for
15 *Mycoplasma pneumoniae*, the probe was tested in hybridization reactions under the following conditions. 32 P-end-labelled oligonucleotide probe was mixed with 1 microgram (7×10^{-13} moles) of purified rRNA from *Mycoplasma pneumoniae* and reacted in 0.12 M PB
20 (equimolar amounts of Na_2HPO_4 and NaH_2PO_4), 1 mM EDTA and 0.2% SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was mixed with the hybridization buffer with and without target *Mycoplasma*
25 *pneumoniae* rRNA present. Following separation on hydroxyapatite as outlined previously the hybrids were quantitated by scintillation counting. These results are shown in Table 24.

SUBSTITUTE SHEET

TABLE 24

5	HYBRIDIZATION OF THE <u>M. PNEUMONIAE</u> 5S rRNA DNA PROBE TO HOMOLOGOUS TARGET rRNA*/		
		<u>plus rRNA</u>	<u>minus rRNA</u>
	<u>M. pneumoniae</u> 5S probe	85-95%	0.5%
10	* % Hybridization = $\frac{\text{cpm bound to hydroxyapatite}}{\text{total cpm added to reaction}}$		

15 This data shows that the probe has a high extent of
reaction to its homologous target and very little non-
specific binding to the hydroxyapatite.

Specificity of the M. pneumoniae 5S probe was
tested by mixing the ³²P labelled probe with rRNA
released from cells from other Mycoplasma species. All
20 hybridization assays were carried out as described in
Example 1. Table 25 indicates that the probe is
specific for Mycoplasma pneumoniae and does not react
with any other Mycoplasma species.

25

TABLE 25

	HYBRIDIZATION OF <u>M. PNEUMONIAE</u> PROBE TO OTHER MYCOPLASMA SPECIES		
30	<u>Organism</u>	<u>ATCC#</u>	<u>%Probe Bound</u>
	Acholeplasma laidlawii	14089	3.3
	M. buccale	23636	1.7
	M. capricolum	23205	2.4
	M. columbinsale	33549	1.4
35	M. faucium	25293	1.4
	M. fermentans	15474	1.0
	M. gallisepticum	19610	1.8

SUBSTITUTE SHEET

	M. gallopavonis	33551	1.6
	M. genitalium	3353c	1.7
	M. hominis	14027	1.3
	M. orale	23714	1.8
5	M. pneumoniae	15531	78.0
	M. primatum	15497	1.6
	M. salivarium	23064	0.6
	Spiroplasma mirum		2.3

10

As shown in Table 26, the probe did not react with any other closely related or phylogenetically diverse species of bacteria.

15

TABLE 26

20 HYBRIDIZATION OF M. PNEUMONIAE PROBE TO
A PHYLOGENETIC CROSS SECTION OF BACTERIA

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Corynebacterium xerosis	373	1.4
	Haemophilus influenzae	19418	1.4
	Klebsiella pneumoniae	23357	1.3
25	Legionella pneumophila	33152	1.8
	Mycobacterium tuberculosis (avir)	25177	1.6
	Mycoplasma pneumoniae	15531	52
	Neisseria meningitidis	13077	0.6
30	Propionibacterium acnes	6919	2.0
	Pseudomonas aeruginosa	25330	1.6
	Staphylococcus aureus	12598	2.0
	Streptococcus pneumonia	c6306	1.9

35

Four additional probe sequences (numbered 2-5 below) specific for Mycoplasma pneumoniae were obtained by utilizing four unique primers complementary to conserved regions on 16S rRNA. The regions correspond, respectively, to bases 190-230; 450-490; 820-860; and 1255-1290 of E. coli 16s rRNA. Probe sequence #1 was obtained using a primer with the sequence 5'-

SUBSTITUTE SHEET

CGK00033198

GGCCGTTACCCACCTACTAGCTAAT-3'. Probe sequence #2 was obtained with a primer with the sequence 5'-GTATTACCGCGGCTGCTGGC-3'. Probe sequence #3 was obtained with a primer with the sequence 5'-
5 CCGCTTGTGCGGGCCCCGTCAATTC-3'. Probe sequence #4 was obtained using a primer with the sequence 5'-CGATTACTAGCGATTCC-3'. Sequencing reactions were performed as outlined in previous examples. The M. pneumoniae sequences were compared with sequences from
10 Mycoplasma genitalium, Mycoplasma capricolum, Mycoplasma gallisepticum and Spiroplasma mirum.

The following probe sequences were characterized by criteria described in example one of the parent application and were shown to be specific for
15 Mycoplasma pneumoniae:

2. AATAACGAACCCCTTGCAGGTCCTTTCAACTTTGAT
3. CAGTCAAACCTCTAGCCATTACCTGCTAAAGTCATT
4. TACCGAGGGGATCGCCCCGACAGCTAGTAT
5. CTTTACAGATTTGCTCACTTTTACAAGCTGGCGAC.

20 Probe #2 is 35 bases in length and has a T_m of 67°C. Probe #3 is 35 bases in length and has a T_m of 66°C. Probe #4 is 30 bases in length and has a T_m of 69°C. Probe #5 is 35 bases long with a T_m of 66°C.

25 When the four probes were mixed and used in hybridization assays at 60°C in the same manner as previous examples, they were found to be specific for M. pneumoniae. The probes do not cross react with other respiratory pathogens or with any organism representing the bacterial phylogenetic tree (Table 28).

TABLE 27
HYBRIDIZATION OF MYCOPLASMA PNEUMONIAE
PROBES 2-5 TO MYCOPLASMA SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acholeplasma axanthum	27378	0.34
	Acholeplasma laidlawii	14089	0.30
10	Mycoplasma arginini	23838	0.20
	Mycoplasma arthritidis	19611	0.49
	Mycoplasma bovigenitalium	19852	0.18
	Mycoplasma bovis	25523	0.43
	Mycoplasma buccale	23636	0.37
15	Mycoplasma californicum	33451	0.79
	Mycoplasma capricolum	23205	0.38
	Mycoplasma columbinasale	33549	0.54
	Mycoplasma columborale	29258	0.50
	Mycoplasma faucium	25293	0.45
20	Mycoplasma fermentans	15474	0.27
	Mycoplasma gallisepticum	19610	0.25
	Mycoplasma gallopavonis	33551	0.47
	Mycoplasma genitalium	33530	2.5
	Mycoplasma hominis	14027	0.52
25	Mycoplasma hyorhinitis	17981	0.46
	Mycoplasma orale	23714	0.56
	Mycoplasma pneumoniae	15531	34.0
	Mycoplasma primate	15497	0.71
	Mycoplasma pulmonis	19612	0.68
30	Mycoplasma salivarium	23064	0.46
	Spiroplasma citri	29416	0.60
	Spiroplasma mirum	29335	0.52

35

TABLE 28
HYBRIDIZATION OF MYCOPLASMA PNEUMONIAE
PROBES 2-5 WITH OTHER BACTERIA

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Actinomyces israelii	10049	1.0
40	Bacteroides fragilis	23745	1.4
	Bifidobacterium breve	15700	1.0
	Bordetella bronchiseptica	10580	0.9
	Clostridium innocuum	14501	1.0
	Clostridium pasteurianum	6013	0.9
45	Clostridium perfringens	13124	1.1
	Clostridium ramosum	25582	1.0
	Corynebacterium xerosis	373	0.8

	Erysipelothrix rhusiopathiae	19414	1.1
	Escherichia coli	11775	1.0
	Haemophilus influenzae	19418	0.9
	Klebsiella pneumoniae	15531	1.0
5	Lactobacillus acidophilus	4356	1.4
	Legionella pneumophila	33154	0.8
	Listeria monocytogenes	15313	1.2
	Moraxella osloensis	19976	1.1
	Mycobacterium tuberculosis	25177	1.0
10	Neisseria meningitidis	13077	1.0
	Pasteurella multocida	6529	1.6
	Peptococcus magnus	14955	0.9
	Propionibacterium acnes	6919	1.1
	Pseudomonas aeruginosa	25330	1.0
15	Staphylococcus aureus	12600	1.0
	Streptococcus faecalis	19433	1.5
	Streptococcus mitis	9811	1.0
	Streptococcus pneumoniae	6306	1.0
20	Streptococcus pyogenes	19615	1.1

Example 10

The genus Legionella contains 22 species which are all potentially pathogenic for humans. These organisms cause Legionnaires' disease, an acute pneumonia, or Pontiac fever, an acute, non-pneumonic, febrile illness that is not fatal. Legionella species have also been shown to be responsible for nosocomial pneumonia occurring predominantly among immunocompromised patients.

Legionellosis, which includes Legionnaires' disease and Pontiac fever, is diagnosed on the basis of clinical symptoms, either direct or indirect fluorescence antibody tests, and by culture using a buffered charcoal yeast extract (BCYE) agar containing selective antimicrobial agents. There is no single definitive genus test known in the prior art. (See Bergey's Manual of Systematic Bacteriology at page 283, (ed. 1984)). The fluorescent antibody tests are not able to identify all species of Legionella, but only those few for which antibodies exist. The culture method is not definitively diagnostic for Legionella species.

The oligonucleotide sequences described below, when used as probes in a nucleic acid hybridization assay, accurately identify all species of Legionella. This assay is more sensitive than culture or antibody tests and shortens significantly the time of identification and, thus, diagnosis. The assay, therefore, represents a significant improvement over prior diagnostic methods.

Three probe sequences specific for the genus Legionella were obtained by utilizing three unique primers complementary to conserved regions on both 16S and 23S rRNA. Sequence 1 was obtained by using a 16S primer with the sequence 5'-TCT ACG CAT TTC ACC GCT ACA C-3'. Probe sequence 2 was obtained with a 23S primer of sequence 5'-CAG TCA GGA GTA TTT AGC CTT-3'. Probe sequence 3 was obtained with a 16S primer of sequence 5'-GCT CGT TGC GGG ACT TAA CCC ACC AT-3'. Sequencing with these primers was performed as described for previous examples.

The following three sequences were characterized by the criteria described in Example 1 and were shown to be specific for the genus Legionella. The phylogenetically nearest neighbors Escherichia coli, Pseudomonas aeruginosa, Vibrio parahaemolyticus and Acinetobacter calcoaceticus were used as comparisons with sequences from Legionella species.

1. TACCCTCTCCCACTCGAGTCAACCAGTATTATCTGACC
2. GGATTTACGTGTCCCGGCCTACTTGTTTCGGGTGCGTAGTTC
3. CATCTCTGCAAAATTCACCTGTATGTCAAGGGTAGGTAAGG.

Sequence 1, from 16S rRNA, is 40 bases in length and has a T_m of 72°C. Sequence 2, from 23S rRNA, is 42 bases in length and has a T_m of 73°C. Sequence 3, from 16S rRNA, is 40 bases in length and has a T_m of 68°C. These sequences are capable of hybridizing to RNA of the genus Legionella in the regions corresponding respectively to,

630-675 of E. coli 16s rRNA; 350-395 of E. coli 23s rRNA; and 975-1020 of E. coli 16s rRNA. When mixed together the probes had a combined average T_m of 73°C. Analysis on polyacrylamide gels showed that each probe
 5 was the correct length and sequence analysis demonstrated that each was the correct sequence of bases.

When the three probes were mixed and used in a hybridization assay, they were found to be specific for
 10 the genus Legionella (Tables 29 and 30) and did not cross react with other respiratory pathogens or with any selected organism from the phylogenetic tree (Tables 31 and 32). Use of more than one probe, i.e., a mixture of probes, can result in increased assay sensitivity and/or
 15 in an increase in the number of non-viral organisms to be detected.

TABLE 29

20 HYBRIDIZATION OF LEGIONELLA
 PROBES TO HOMOLOGOUS TARGET rRNA

	<u>plus rRNA</u>	<u>minus rRNA</u>
<u>Legionella</u> probe	80%	1.0%

25

TABLE 30

HYBRIDIZATION OF LEGIONELLA
 PROBES TO LEGIONELLA SPECIES

30	<u>Organism</u>	<u>ATCC#</u>	<u>% Probes Bound</u>
	L. anisa	35292	42.0
	L. bozemanii	33217	58.0
	L. cherrii	35252	69.0
	L. dumoffii	33279	57.0
35	L. erythra	CDC#9PlW044C	26.0
	L. feeleii	35303	59.0

SUBSTITUTE SHEET

	L. hackeliae	35250	47.0
	L. jamestowniensis	35298	20.0
	L. jordanis	33623	50.6
	L. longbeachae	33484	48.0
5	L. maceachernii	35300	25.0
	L. micdadei	33704	38.0
	L. oakridgensis	33761	44.0
	L. parisiensis	9060	69.0
	L. pneumophila 1*	6736	75.0
10	" 2		64.0
	" 3		73.0
	" 4		73.0
	" 5		78.0
	" 6		75.0
15	" 7		73.0
	" 8		63.0
	" 11		75.0
	L. rubrilucens	35304	12.0
	L. sainthelensi	35248	61.0
20	L. sainticrucis	35301	24.0
	L. spiritensis	CDC#MSH9	55.0
	L. steigerwaltii	7430	56.0
	L. wadsworthii	33877	37.0
25	* The numbers 1-8 and 11 are serotypes of <u>L. pneumophila</u> .		

TABLE 31

30 HYBRIDIZATION OF LEGIONELLA PROBES TO
RESPIRATORY PATHOGENS

	<u>Organisms</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Corynebacterium xerosis	373	2.1
	Haemophilus influenzae	19418	2.3
35	Klebsiella pneumoniae	23357	2.0
	Mycoplasma pneumoniae	15531	2.3
	Neisseria meningitidis	13090	2.2
	Pseudomonas aeruginosa	25330	1.2
	Propionibacterium acnes	6919	1.6
40	Streptococcus pneumoniae	6306	0.8
	Staphylococcus aureus	25923	1.6

TABLE 32

HYBRIDIZATION OF LEGIONELLA PROBES TO
A PHYLOGENETIC CROSS SECTION
OF BACTERIAL SPECIES

5

	<u>Organisms</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter calcoaceticus	33604	1.4
	Branhamella catarrhalis	25238	2.0
10	Bacillus subtilis	6051	1.9
	Bacteroides fragilis	23745	2.2
	Campylobacter jejuni	33560	1.2
	Chromobacterium violaceum	29094	1.3
	Clostridium perfringens	13124	1.9
15	Deinococcus radiodurans	35073	1.8
	Derxia gummosa	15994	2.0
	Enterobacter aerogenes	13048	1.4
	Escherichia coli	11775	1.2
	Mycoplasma hominis	14027	1.1
20	Proteus mirabilis	29906	1.4
	Pseudomonas cepacia	11762	1.1
	Rahnella aquatilis	33071	1.7
	Rhodospirillum rubrum	11170	2.0
	Streptococcus mitis	9811	2.0
25	Vibrio parahaemolyticus	17802	2.0
	Yersinia enterocolitica	9610	1.2

30 Three additional probe sequences (numbered 4-6)
specific for the genus Legionella were obtained by
utilizing two primers complementary to conserved regions
on 23S rRNA. Sequence 4 was made from a 23S primer with
the sequence 5'-CCT TCT CCC GAA GTT ACG G-3'. Probe
35 sequences 5 and 6 were made from a 23S primer of
sequence 5'-AAG CCG GTT ATC CCC GGG GTA ACT TTT-3".
Sequencing with these primers was performed as described
for previous examples.

40 The following three sequences were characterized by
the criteria previously described and were shown to be
specific for the genus Legionella. The
phylogenetically nearest neighbors Escherichia coli,

SUBSTITUTE SHEET

Pseudomonas aeruginosa, Vibrio parahaemolyticus and Actinetobacter calcoaceticus were used for comparisons with sequences from Legionella species.

4. GCG GTA CGG TTC TCT ATA AGT TAT GGC TAG C

5. GTA CCG AGG GTA CCT TTG TGC T

6. CAC TCT TGG TAC GAT GTC CGA C

Probe 4, complementary to 23S rRNA in the region corresponding to bases 1585-1620 of E. coli 23s rRNA, is 31 bases long and has a T_m of 67°C. Probe 5, complementary to 23S rRNA in the region corresponding to bases 2280-2330 of E. coli 23s rRNA, is 22 bases long and has a T_m of 66°C. Probe 6, complementary to 23S rRNA in the same region as Probe 5, is 22 bases long and has a T_m of 63°C.

When the three probes were mixed with probe 3 above and used in a hybridization assay as described for probes 1-3, they were found to be specific for the genus Legionella (Table 33) and did not cross react with other respiratory pathogens or with any selected organism from the phylogenetic tree (Tables 34 and 35). Using more than one probe, i.e., a mixture of probes, can improve assay sensitivity and/or increase the number of non-viral organisms detected.

TABLE 33

HYBRIDIZATION OF LEGIONELLA PROBES TO
LEGIONELLA SPECIES

Organism	ATCC#	% Probes Bound
L. anisa	35292	29.6
L. bozemanii	33217	35.5
L. cherrii	35252	29.2
L. dumoffii	33279	26.0
L. erythra	35303	32.0
L. feelii	CDC#9P1W044C	32.0
L. hackeliae	35250	39.0

	L. jamestowniensis	35298	31.2
	L. jordanis	33623	25.7
	L. longbeachae	33484	27.6
	L. maceahernii	35300	39.3
5	L. micdadei	33204	31.0
	L. oakridgensis	33761	24.4
	L. parisiensi	35299	31.2
	L. pneumophila 1*	33153	40.0
	" 2	33154	38.5
10	" 3	33155	44.6
	" 4	33156	48.6
	" 5	33216	32.0
	" 6	33215	43.0
	" 7	33823	29.5
15	" 8	35096	37.6
	" 11	43130	44.5
	L. rubrilucens	35304	30.1
	L. sainthelensis	35248	27.0
	L. sainticrucis	35301	22.0
20	L. spiritensis	CDC#MSH9	40.5
	L. steigerwalti	35302	31.7
	L. wadsworthii	33877	30.0

* The numbers 1-8 and 11 are serotypes of L. pneumophila.

25

TABLE 34

HYBRIDIZATION OF LEGIONELLA PROBES TO
RESPIRATORY PATHOGENS

30	<u>Organisms</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Corynebacterium xerosis	373	0.13
	Haemophilum influenzae	19418	0.12
	Klebsiella pneumoniae	23357	0.13
	Neisseria meningitidis	13090	0.14
35	Pseudomonas aeruginosa	25330	0.13
	Propionibacterium acnes	6919	0.11
	Streptococcus pneumoniae	6306	0.08
	Staphylococcus aureus	25923	0.15

40

SUBSTITUTE SHEET

TABLE 35

HYBRIDIZATION OF LEGIONELLA PROBES TO
A PHYLOGENETIC CROSS SECTION OF BACTERIAL SPECIES

	<u>Organisms</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
5	Acinetobacter calcoaceticus	33604	0.12
	Branhamella catarrhalis	25238	0.13
	Bacillus subtilis	6051	0.09
	Bacteroides fragilis	23745	0.12
10	Campylobacter jejuni	33560	0.06
	Chromobacterium violaceum	29094	0.33
	Clostridium perfringens	13124	0.07
	Deinococcus radiodurans	35073	0.11
	Derxia gummosa	15994	0.15
15	Enterobacter aerogenes	13048	0.26
	Escherichia coli	11775	0.09
	Mycoplasma hominis	14027	0.09
	Proteus mirabilis	29906	0.09
	Pseudomonas cepacia	17762	0.20
20	Rahnella aquatilis	33071	0.15
	Rhodospirillum rubrum	11170	0.13
	Streptococcus mitis	9811	0.07
	Vibrio parahaemolyticus	17802	0.11
25	Yersinia enterocolitica	9610	0.19

Example 11

Chlamydia are gram-negative, non-motile, obligate intracellular bacteria. The species C. trachomatis is associated with endemic trachoma (the most common preventable form of blindness), inclusion conjunctivitis and lymphogranuloma venereum (LGV). It is a major cause of nongonococcal urethritis in men and may cause cervicitis and acute salpingitis in women. Eye disease or chlamydial pneumonia may develop in newborns passing through the infected birth canal.

There are several methods known in the art for identification of C. trachomatis in the urogenital tract, for example, by direct immunofluorescent staining

or enzyme immunoassay of clinical specimens. The method of choice, however, remains culture of the organism in cycloheximide treated McCoy cells. Cell culture is followed by morphological or fluorescent antibody staining for confirmation of the organism's identity.

The inventive oligonucleotide sequences described below, when used as probes in nucleic acid hybridization assay, accurately identify Chlamydia trachomatis isolates. This assay test is equal in sensitivity to culture or antibody tests and, in the case of culture, significantly shortens the time to identification, and thus, diagnosis.

The use of probes to identify and distinguish between members of the species is novel and inventive. Indeed, Kingsbury, D.T., and E. Weiss, 1968 J. Bacteriol. 96: 1421-23 (1968); Moulder, J.W., ASM News, Vol.50, No.8, (1984) report a 10% DNA homology between C. trachomatis and C. psittaci. Moreover, these reports show that different C. trachomatis strains differ in DNA homology. Weisberg, W.G. et. al, J. Bacteriol. 167:570-574 (1986) published the 16S rRNA sequences of C. psittaci and noted that C. trachomatis and C. psittaci share a greater than 95% rRNA homology. From these reports, it may be inferred that it would be difficult to invent (1) probes capable of hybridizing to all strains of C. trachomatis; and (2) probes capable of distinguishing between C. trachomatis and C. psittaci. The following probes accomplish both objectives.

Ten probe sequences specific for Chlamydia trachomatis were made using seven unique primers complementary to conserved regions of both 16S and 23S rRNA. Probe sequence 1 was obtained from a 16S primer of sequence 5'-TCT ACG CAT TTC ACC GCT ACA C-3'. Probe sequence 2 was obtained with a 16S primer of sequence 5'-CCG CTT GTG CGG GCC CCC GTC AAT TC-3'. Sequences 3

and 4 were obtained using a 16S primer with the sequence 5'-GGC CGT TAC CCC ACC TAC TAG CTA AT-3'. Probe sequences 5 and 6 were obtained with a 23S primer of sequence 5'-CTT TCC CTC ACG GTA-3'. Probe sequences 7 and 8 were obtained with a 23S primer of sequence 5'-CCT TCT CCC GAA GTT ACG G-3'. Probe sequence 9 was obtained with a 23S primer of sequence 5'-TCG GAA CTT ACC CGA CAA GGA ATT TC-3'. Probe sequence 10 was obtained with a primer of sequence 5'-CTA CTT TCC TGC GTC A-3'.

The following ten sequences were characterized using the criteria described in Example 1 and were shown to be specific for the rRNA of Chlamydia trachomatis. The phylogenetically nearest neighbor Chlamydia psittaci was used for comparison with Chlamydia trachomatis sequence.

1. CCG ACT CGG GGT TGA GCC CAT CTT TGA CAA
2. TTA CGT CCG ACA CGG ATG GGG TTG AGA CCA TC
3. CCG CCA CTA AAC AAT CGT CGA AAC AAT TGC TCC
- 20 GTT CGA
4. CGT TAC TCG GAT GCC CAA ATA TCG CCA CAT TCG
5. CAT CCA TCT TTC CAG ATG TGT TCA ACT AGG AGT
- CCT GAT CC
6. GAG GTC GGT CTT TCT CTC CTT TCG TCT ACG
- 25 7. CCG TTC TCA TCG CTC TAC GGA CTC TTC CAA TCG
8. CGA AGA TTC CCC TTG ATC GCG ACC TGA TCT
9. CCG GGG CTC CTA TCG TTC CAT AGT CAC CCT AAA AG
10. TAC CGC GTG TCT TAT CGA CAC ACC CGC G

Sequence 1, from 16S rRNA, is 30 bases in length and has a T_m of 66°C. Sequence 2, from 16S rRNA, is 32 bases in length and has a T_m of 67°C. Sequence 3, from 16S rRNA, is 39 bases in length and has a T_m of 70°C. Sequence 4, from 16S rRNA, is 33 bases in length and has a T_m of 69°C. Sequence 5, from 23S rRNA, is 41 bases in length and has a T_m of 71°C. Sequence 6, from 23S rRNA,

SUBSTITUTE SHEET

is 30 bases in length and has a T_m of 72°C. Sequence 7, from 23S rRNA, is 33 bases in length and has a T_m of 72°C. Sequence 8, from 23S rRNA, is 30 bases in length and has a T_m of 71°C. Sequence 9, from 23S rRNA is 35
5 bases in length and has a T_m of 74°C. Sequence 10 is 28 bases in length and has a T_m of 72°C.

The reactivity and specificity of the probes was tested hybridization assays. ³²P-end-labeled oligonucleotide probes 1 and 2 were mixed with purified
10 RNA or RNA released from at least 10⁷ organisms in 0.55 ml of 41% diisobutyl sulfosuccinate, 3% sodium dodecyl sulfate, 0.03 M sodium phosphate pH 6.8, 1mM EDTA, 1mM EGTA at 60°C (probe 1) or 64°C (probe 2) for 1 hour. Hybrids were bound to hydroxyapatite as described in
15 previous examples and the amount of radioactivity bound was determined by scintillation counting. Table 36 shows that probes 1 and 2 hybridize well to all serotypes of C. trachomatis tested. Probe 1 does not react with any strain of C. psittaci tested and probe 2
20 does not react with two of the strains. Probe 2 does react with the ovine polyarthrititis strain of C. psittaci, an organism which is not known to infect humans. Table 37 demonstrates the reactivity and specificity of probes 3-9 when ¹²⁵I-labeled and used as
25 a mix. In this case, the hybrids were bound to cationic magnetic particles as described in Arnold et al., U.S. Patent App. Ser. No. 020,866 filed March 2, 1987. These probes hybridize well to all strains of C. trachomatis tested and not to any strains of C. psittaci. Probes 3-
30 9 were further tested against a panel of organisms commonly found in the urogenital tract (Table 38) and a phylogenetic cross section of organisms (Table 39). In all cases, the probes were shown to be specific. Probe 10 is 25% non-homologous to C. psittaci and also should
35 be specific for C. trachomatis.

TABLE 36

5 HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 1 AND 2
TO CHLAMYDIA RNA

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>	
			<u>Probe 1</u>	<u>Probe 2</u>
10	Chlamydia trachomatis serotype C	VR578	22	39
	Chlamydia trachomatis serotype E	VR348B	27	48
	Chlamydia trachomatis serotype G	VR878	20	44
15	Chlamydia trachomatis serotype I	VR880	20	42
	Chlamydia trachomatis serotype K	VR887	28	45
	Chlamydia psittaci			
20	guinea pig conjunctivitis strain	VR813	1.2	1.4
	Chlamydia psittaci ovine abortion strain	VR656	1.0	3.0
	Chlamydia psittaci ovine			
25	poly-arthritis strain	VR619	1.1	35.3

TABLE 37

30 HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 3-9
WITH CHLAMYDIA rRNA

35	<u>Organism</u>	<u>Ratio Counts</u>		<u>ATCC#</u>	<u>Bound*</u>
		<u>Serovar</u>			
	C. trachomatis	A			689
	C. trachomatis	B			560
	C. trachomatis	Ba			1066
	C. trachomatis	C	VR548		962
40	C. trachomatis	D			1192
	C. trachomatis	E	VR348		1022
	C. trachomatis	F			391
	C. trachomatis	G	VR878		874
	C. trachomatis	H			954
45	C. trachomatis	I	VR880		943
	C. trachomatis	J			482
	C. trachomatis	K	VR887		999

SUBSTITUTE SHEET

	C. trachomatis	L1		638
	C. trachomatis	L2		501
	C. trachomatis	L3	VR903	821
	C. psittaci		VR125	1.6
5	C. psittaci		VR629	0.9
	C. psittaci		VR656	1.3
	C. psittaci		VR813	1.2

*Ratio = $\frac{\text{counts bound when RNA present}}{\text{counts bound when no RNA present}}$

10

TABLE 38

15 HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 3-9
TO ORGANISMS FOUND IN THE UROGENITAL TRACT

	<u>Organism</u>	<u>ATCC#</u>	<u>Ratio Counts Bound*</u>
	Achromobacter xylosoxidans	27061	1.9
	Acinetobacter lwoffii	15309	1.2
20	Branhamella catarrhalis	25238	1.2
	Candida albicans	18804	2.4
	Flavobacterium meningosepticum	13253	1.1
	Gardnerella vaginalis	14018	1.3
25	Lactobacillus acidophilus	4356	0.8
	Listeria monocytogenes	15313	0.7
	Mycobacterium smegmatis	14468	1.1
	Moraxella osloensis	19976	1.3
	Neisseria gonorrhoeae	19424	2.3
30	Pasteurella multocida	6529	1.0
	Peptostreptococcus anaerobius	27337	1.2
	Streptococcus agalactiae	13813	4.0
	Streptococcus faecalis	19433	2.6

35 *Ratio = $\frac{\text{counts bound when RNA present}}{\text{counts bound when no RNA present}}$

SUBSTITUTE SHEET

TABLE 39

5 HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 3-9
 TO PHYLOGENETICALLY DIVERSE ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u>Ratio Counts</u> <u>Bound*</u>
	Bacillus subtilis	6051	2.2
10	Bacteroides fragilis	23745	1.6
	Campylobacter jejuni	33560	1.4
	Chromobacterium violaceum	29094	1.4
	Deinococcus radiodurans	35073	1.8
15	Derxia gummosa	15994	1.3
	Enterobacter aerogenes	13048	1.9
	Escherichia coli	11775	1.9
	Mycoplasma hominis	14027	1.3
	Pseudomonas cepacia	17762	2.2
20	Proteus mirabilis	29906	2.2
	Rahnella aquatilis	33071	1.9
	Rhodospirillum rubrum	11170	1.9
	Vibrio parahaemolyticus	17802	2.0
	Yersinia enterocolitica	9610	2.5
25	*Ratio = <u>counts bound when RNA present</u> <u>counts bound when no RNA present</u>		

30 Example 12

 Campylobacters are motile, microaerophilic, gram
 negative curved rods. The genus is quite diverse and
 distinct from other genera. Although the genus is well
 defined, some revision is occurring at the species
 35 level (Romaniuk, P.J. et al., J. Bacteriol. 169:2137-
 2141 (1987). Three Campylobacter species, Campylobacter
jejuni, C. coli and C. laridis, cause enteritis in
 humans. The disease includes diarrhea, fever, nausea,
 abdominal pain and in some cases, vomiting. These
 40 organisms cause an estimated 2 million infections per
 year in the United States (estimate based on the number

SUBSTITUTE SHEET

of Salmonella and Shigella induced cases of diarrheal disease). Other members of the genus cause septicemias in humans and abortion and infertility in sheep and cattle.

5 Diagnosis of Campylobacter enteritis is currently dependent upon growth and isolation of the organism in culture, followed by a number of biochemical tests. Optimum growth of campylobacters requires special conditions such as low oxygen tension and high
10 temperature (42°C). No single set of conditions is recommended for isolation of all Campylobacter species.

 The oligonucleotide sequences listed below, when used in a hybridization assay, hybridize to the 16S rRNA of the Campylobacter species of interest. The
15 present invention has significant advantages over the prior art methods of detection of Campylobacter because one probe can detect all Campylobacters of interest; the other two probes detect the enteric Campylobacters and one can detect human isolates of Campylobacter. In
20 addition, the probes have advantages over the prior art in terms of ease of the assay and greatly reduced time to identification and therefore, diagnosis.

 The four probes which hybridize to the 16S rRNA of Campylobacter species of interest were constructed using
25 three unique primers complementary to 16S rRNA. Sequences 1 and 2 were made using a 16S primer with the sequences 5'-GTA TTA CCG CGG CTG CTG GCA C-3'. Sequence 3 was made using a 16S primer with the sequence 5'-CCG CTT GTG CGG GCC CCC GTC AAT TC-3'. Sequence 4 was made
30 with a 16S primer with the sequence 5'-GCT CGT TGC GGG ACT TAA CCC AAC AT-3'.

 The following sequences were characterized and shown to hybridize to Campylobacter jejuni, C. coli and C. laridis. The phylogenetically nearest neighbors

SUBSTITUTE SHEET

CGK00033215

Vibrio parahaemolyticus and Wollinella succinogenes were used for comparison with the campylobacter sequences.

1. CGC TCC GAA AAG TGT CAT CCT CC
2. CCT TAG GTA CCG TCA GAA TTC TTC CC
- 5 3. GCC TTC GCA ATG GGT ATT CTT GGT G
4. GGT TCT TAG GAT ATC AAG CCC AGG

Sequence 1, from 16S rRNA, is 23 bases in length and has a T_m of 65°C. Sequence 2, from 16S rRNA, is 26 bases in length and has a T_m of 64°C. Sequence 3, from 10 16S rRNA, is 25 bases in length and has a T_m of 66°C. Sequence 4, from 16S rRNA, is 24 bases in length and has a T_m of 61°C. Sequence 1 is capable of hybridizing in the region corresponding to bases 405-428 of E. coli 16s rRNA; Sequence 2 is capable of hybridizing in the region 15 corresponding to bases 440-475 of E. coli 16s rRNA; Sequence 3 is capable of hybridizing in the region corresponding to bases 705-735 of E. coli 16s rRNA; Sequence 4 is capable of hybridizing in the region corresponding to bases 980-1010 of E. coli 16s rRNA.

20 The reactivity and specificity of the probes for campylobacter was tested in hybridization assays. ³²P-end-labeled oligonucleotide probes were mixed with purified RNA or RNA released from cells in 0.1% sodium dodecyl sulfate. 0.5 ml of hybridization solution (41% 25 diisobutyl sulfosuccinate, 30mM sodium phosphate, pH 6.8, 0.7% sodium dodecyl sulfate, 1mM EDTA, 1mM EGTA) was added and the mixture incubated at 60°C for 1 to 1.5 hour. Following incubation, 2 to 2.5 ml of separation solution (2% hydroxyapatite, 0.12 M sodium 30 phosphate, pH6.8, 0.02% sodium dodecyl sulfate) was added and the mixture incubated at 60°C for five minutes. The sample was centrifuged and the supernatant removed. 2.5 ml of wash solution (0.12 M sodium phosphate, pH6.8, 0.02% sodium dodecyl sulfate) 35 was added and the sample mixed, centrifuged and the

supernatant removed. The radioactivity bound to the hydroxyapatite was determined by scintillation counting.

Table 40 indicates that the probes hybridize well to the Campylobacter species of interest, C. jejuni, C. coli, and C. laridis. Probe 1 detects all of the Campylobacter species tested, probes 2 and 4 detect only the enteric campylobacters, and probe 3 detects all of the Campylobacter species except C. sputorum, an organism isolated from cattle. Thus all of the probes are useful for identifying campylobacter in stool samples. The choice of which probe to use for other applications would depend upon the level of specificity required (i.e., enteric campylobacters, or all Campylobacter species).

TABLE 40

HYBRIDIZATION OF CAMPYLOBACTER PROBES 1-4
TO CAMPYLOBACTER SPECIES

Organism	ATCC#	% Probe Bound (*)			
		1	2	3	4
Campylobacter coli	33559	64	70	52	49
C. fetus					
subsp. fetus	27374	68	0.1	66	0.5
C. fetus					
subsp. venerealis	19438	66	0.7	54	1.2
C. jejuni	33560	63	76	51	56
C. laridis	35221	74	73	64	52
C. sputorum					
subsp. bubulus	33562	71	3.0	2.5	0

(*) % Probe Bound = cpm bound to hydroxyapatite-cpm bound when no RNA present/total cpm used in the assay

SUBSTITUTE SHEET

Table 41 shows that the probes do not hybridize to closely related organisms or organisms found in the gastrointestinal tract.

5

TABLE 41

HYBRIDIZATION OF CAMPYLOBACTER PROBES 1-4 TO CLOSELY RELATED ORGANISMS AND ORGANISMS FOUND IN THE GASTRO-INTESTINAL TRACT.

10	Organism	ATCC#	% Probe Bound (*)			
			1	2	3	4
	Bacteroides fragilis	25285	0	0.2	0.7	0
15	Escherichia coli	11775	1.3	0.5	0.5	0
	Salmonella typhimurium	14028	0	0	0.3	0
	Shigella boydii	29929	0	0.2	0.5	0
20	Shigella dysenteriae	13313	0	0.7	0.2	0
	Shigella flexneri	29903	0	0	0.5	0
25	Shigella sonnei	29930	0	0	0.1	0
	Vibrio parahae-					
	molyticus	17802	0	1.9	0.1	0
30	Wollinella succinogenes	29543	0.4	2.1	2.2	0
	Yersinia pseudotuberc-					
	ulosis	29833	0.6	0.2	1.7	0.3

*) % probe bound = cpm bound to hydroxyapatite-cpm und when no RNA present/total cpm used in the assay

40 The probes specific for the enteric Campylobacters, probes 2 and 4, were further tested and shown not to react with rRNAs of other organisms found in the gastrointestinal tract.

SUBSTITUTE SHEET

CGK00033218

TABLE 42

HYBRIDIZATION OF CAMPYLOBACTER PROBES 2 AND 4 TO
ORGANISMS FOUND IN THE GASTROINTESTINAL TRACT.

	Organism	ATCC#	% Probe Bound (*)	
			Probe 2	Probe 4
	Citrobacter diversus	27156	0	0
	Clostridium perfringens	13124	0	0
10	Enterobacter cloacae	13047	0	0
	Klebsiella pneumoniae	23357	0	0.5
	Proteus mirabilis	25933	0	0
	Serratia marcescens	13880	0	0
	Staphylococcus aureus	e12600		
15	Staphylococcus			
	epidermidis	14990	0	0.3
	Streptococcus bovis	33317	0	0

(*) % probe bound = cpm bound to hydroxyapatite-cpm
bound when no RNA present/total cpm used in the
assay

Example 13

Streptococci are gram positive, oxidase negative
coccoid bacteria. The genus has been divided into 18
groups, A-R, on the basis of group-specific
carbohydrates. Group D streptococci are further
subdivided into the enterococci (S. faecium, S.
30 faecalis, S. avium and S. gallinarum and the non-
enterococci S. bovis and S. equinus. S. faecium, S.
faecalis and S. avium are considered the medically
important enterococci. Some species of streptococcus
are human pathogens; others are normal flora in the
35 mouth and intestine but are capable of causing disease
when introduced to other sites. Two examples are S.
faecium and S. faecalis which are normally found in the

SUBSTITUTE SHEET

intestine but may spread to cause bacteremia, wound infections, and as many as 10% of the urinary tract infections in the United States.

Current methods of detection of enterococci
5 require culture of the specimen for 18-72 hours followed by a battery of biochemical tests. The oligonucleotide sequence shown below, when used in a hybridization assay, accurately detects Streptococcus faecalis, S. avium, and S. faecium. The inventive probe does not
10 cross react with other Streptococci or Staphylococci which are very closely related in DNA homology. (Kiepper-Baez, 1981, 1982, Schliefer 1984.) The current invention also reduces the number of tests which must be run on a sample and greatly reduces the time to
15 identification and thus, diagnosis. This represents a significant improvement over prior art methods.

The probe sequence was identified using a primer complementary to 16S rRNA with the sequence 5'-CCG CTT GTG CGG GCC CCC GTC AAT TC-3'. The following sequence
20 was characterized and shown to be specific for three enterococci, S. faecium, S. faecalis and S. avium. The phylogenetically nearest neighbors S. agalactiae, S. bovis, S. pneumoniae and S. pyogenes were used for comparison with the sequences of interest.

25 1. TGC AGC ACT GAA GGG CGG AAA CCC TCC AAC ACT TA

The sequence is 35 bases in length and has a T_m of 72°C. It is capable of hybridizing in the region corresponding to bases 825-860 of E. coli 16S rRNA. To demonstrate the reactivity and specificity of the probe,
30 it was used in a hybridization assay with purified RNA or RNA released from cells. A suspension containing at least 10⁷ cells in 2% sodium dodecyl sulfate was vortexed in the presence of glass beads. 0.1 ml of suspension was mixed with 0.1 ml of hybridization buffer
35 (0.96 M sodium phosphate, pH 6.8, 0.002 M EDTA, 0.002 M

EGTA) and incubated at 65°C for 2 hours. After incubation, 5 ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added and the mixture was incubated at 65°C for 10 minutes.

5 The sample was centrifuged and the supernatant removed. Five ml of wash solution (0.12 M phosphate buffer, pH 6.8, 0.02% sodium dodecyl sulfate) was added and the samples were vortexed, centrifuged, and the supernatant removed. The amount of radioactivity bound to the

10 hydroxyapatite was determined by scintillation counting. Table 43 shows that the probe reacts well with S. faecium, S. faecalis, and S. avium, and does not react with other closely related organisms.

15

TABLE 43

HYBRIDIZATION OF THE ENTEROCOCCUS PROBE
TO CLOSELY RELATED ORGANISMS.

20	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Staphylococcus aureus	12600	1.4
	Streptococcus agalactiae	13813	1.5
	Streptococcus avium	14025	22.7
	Streptococcus bovis	33317	1.4
25	Streptococcus faecalis	19433	45.3
	Streptococcus faecium	19434	43.0
	Streptococcus mitis	9811	1.5
	Streptococcus pneumoniae	6306	1.5
30	Streptococcus pyogenes	19615	1.3

Example 14

Pseudomonads are gram-negative, nonsporeforming,

35 nonfermentative bacilli. Pseudomonads are common inhabitants of soil and water and rarely infect healthy individuals. When the organisms encounter already

SUBSTITUTE SHEET

compromised patients, they can cause a variety of clinical syndromes including wound infections, post-surgical infections, septicemia, infant diarrhea and respiratory and urinary tract infections. Members of the genus Pseudomonas are particularly important to identify in a clinical sample because of the resistance of the organisms to antibiotics. Nucleic acid homology studies have divided the genus into five homology classes known as RNA groups I-V. Eighty-three percent of all clinical isolates of Pseudomonas are from RNA group I and Pseudomonas aeruginosa is by far the most common species isolated.

Current methods of detection of pseudomonas require culture of a patient sample for 24-72 hours, followed by a battery of biochemical tests. The oligonucleotide sequence below, when used in a hybridization assay, detects the clinically important group I pseudomonas. The present invention reduces the number of tests which must be run on a sample, and reduces the time to detection. This represents a significant improvement over prior art methods.

The sequence was obtained with a primer complementary to a conserved region on 23S rRNA with the sequence 5'-CTT TCC CTC ACG GTA-3'. The following sequence was shown to detect group I pseudomonads:

1. CAG ACA AAG TTT CTC GTG CTC CGT CCT ACT CGA TT

The probe is 35 bases in length and has a T_m of 70°C. It is capable of hybridizing to the RNA of group I Pseudomonas in the region corresponding to bases 365-405 of E. coli 23s rRNA. To demonstrate the reactivity and specificity of the probe, it was used in a hybridization assay. ^{32}P -end-labeled oligonucleotide was mixed with RNA released from at least 10^7 organisms by standard methods in 0.48 M sodium phosphate pH 6.8,

SUBSTITUTE SHEET

CGK00033222

1% sodium dodecyl sulfate, 1 mM EDTA, 1 mM EGTA and incubated at 65°C for two hours. After incubation, the RNA:DNA hybrids were bound to hydroxyapatite as described for previous examples and the radio-activity bound was determined by scintillation counting. Table 44 demonstrates that the probe reacted well with all 8 species of group I pseudomonads that were tested. The probe did not react with RNA from group II or group V organisms. A low reaction was seen with Pseudomonas acidovorans, a group III organism which re-presents < 1% of all isolates of nonfermentative bacilli from clinical samples. Table 45 demonstrates that the probe does not react with other closely related organisms which were tested.

TABLE 44

HYBRIDIZATION OF PSEUDOMONAS GROUP I
PROBE TO PSEUDOMONAS RNAs

<u>Organism</u>	<u>Group</u>	<u>ATCC#</u>	<u>% Probe* Bound</u>
Pseudomonas alcaligenes	I	14909	24
Pseudomonas aeruginosa	I	10145	83
25 Pseudomonas denitrificans	I	13867	83
Pseudomonas fluorescens	I	13525	82
Pseudomonas mendocina	I	25411	79
Pseudomonas pseudoalcaligenes	I	17440	78
30 Pseudomonas putida	I	12633	80
Pseudomonas stutzeri	I	17588	84
Pseudomonas cepacia	II	25416	0
Pseudomonas pickettii	II	27511	1.0
Pseudomonas acidovorans	III	15668	11
35 Pseudomonas maltophilia	V	13637	0.2

*% Probe Bound = counts bound when RNA present - counts bound
when no RNA present/total counts used in the assay

SUBSTITUTE SHEET

CGK00033223

TABLE 45

HYBRIDIZATION OF PSEUDOMONAS GROUP I
 PROBE TO RNAs OF CLOSELY RELATED ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	% Probe* <u>Bound</u>
	Acinetobacter calcoaceticus	23055	1.6
	Legionella pneumophila	33155	0.6
10	Moraxella phenylpyruvica	23333	0.3
	Morganella morganii	25830	0
	Vibrio parahaemolyticus	17802	0.6
	*% Probe Bound = counts bound when RNA present - counts bound when no RNA present/total counts used in the assay		

Example 15

Examples 15-18 disclose probes for the Enterobacteriaceae, all of which are highly related at the DNA level. Even fewer differences exist at the rRNA level. For example, Proteus vulgaris 16s rRNA is 93% homologous to E. coli. These factors illustrate the difficulties associated with making rRNA probes specific for this group of organisms. Nevertheless, we have invented probes for Enterobacter cloacae, Proteus mirabilis, Salmonella and E. coli.

Members of the genus Enterobacter are motile, gram negative, non-sporeforming bacilli which belong in the family Enterobacteriaceae. The genus is a large and heterogeneous group. Eight species have been defined but only 5 are clinically significant. Enterobacter cloacae and E. aerogenes are the most common isolates and are associated with genitourinary, pulmonary, blood, central nervous system and soft tissue infections in humans.

The current method for identifying Enterobacter cloacae from patient samples involves culture of the specimen on agar plates for 18-24 hours, followed by a battery of biochemical tests. The oligonucleotide sequence described below, when used as a probe in a nucleic acid hybridization assay, accurately identifies Enterobacter cloacae. The present invention reduces the number of tests which must be run on a sample, the time to identification and therefore, diagnosis, and thus represents a significant improvement over prior art methods.

The probe specific for Enterobacter cloacae was obtained with a primer complementary to a conserved region of 23S rRNA with the sequence 5'-CAG TCA GGA GTA TTT AGC CTT-'3.

The following sequence was characterized and shown to be specific for E. cloacae. The phylogenetically nearest neighbors Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Salmonella enteritidis, and Citrobacter freundii were used as comparisons with the sequence of E. cloacae.

1. GTG TGT TTT CGT GTA CGG GAC TTT CAC CC

The probe is 29 bases in length and has a T_m of 68°C. It is capable of hybridizing to RNA of E. cloacae in the region corresponding to bases 305-340 of E. coli 23s rRNA. To demonstrate the reactivity and specificity of the probe for E. cloacae, it was used in a hybridization assay. ^{32}P -end-labeled oligonucleotide probe was mixed with RNA released from at least 10^7 organisms in 1% sodium dodecyl sulfate, 0.48 M sodium phosphate, pH 6.8 (0.2 ml final volume) and incubated at 60°C for 2 hours. Following incubation, 5 ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added and the mixture incubated at 60°C for 10 minutes. The sample was

SUBSTITUTE SHEET

CGK00033225

centrifuged and the supernatant removed. Five ml of wash solution (0.12 M sodium phosphate, pH 6.8, 0.02% sodium dodecyl sulfate) was added, the sample vortexed, centrifuged and the supernatant removed. The amount of radioactivity bound to the hydroxyapatite was determined by scintillation counting. The results are shown in Table 46 and demonstrates that the probe reacts well with E. cloacae and does not react with the RNA of closely related organisms.

TABLE 46

HYBRIDIZATION OF ENTEROBACTER CLOACAE PROBE
TO CLOSELY RELATED ORGANISMS

	<u>Organisms Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Citrobacter freundii	8090	1.8
	Enterobacter aerogenes	13048	1.4
	Enterobacter cloacae	13047	27.
20	Escherichia coli	11775	1.0
	Klebsiella pneumoniae	13883	1.7
	Proteus mirabilis	29906	0.9
	Proteus vulgaris	13315	0.6
25	Providencia stuartii	29914	1.1

Table 47 shows that the probe does not react with the RNA of organisms found in urine.

TABLE 47

HYBRIDIZATION OF ENTEROBACTER CLOACAE
PROBE TO ORGANISMS FOUND IN URINE.

	<u>Organisms Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
35	Candida albicans	18804	0.8
	Candida krusei	34135	0.8

SUBSTITUTE SHEET

	Candida parapsilosis	22019	0.9
	Candida tropicalis	750	1.1
	Pseudomonas aeruginosa	10145	1.0
	Serratia marcescens	13880	1.6
5	Staphylococcus aureus	12600	1.7
	Staphylococcus epidermidis	14990	1.4
	Streptococcus agalactiae	13813	2.5
	Streptococcus faecium	19434	1.5
10	Torulopsis glabrata	2001	0.9

Example 16

15 Members of the genus Proteus are motile, gram negative, non-sporeforming bacilli which belong in the family Enterobac-teriaceae. Four species of Proteus have been described and three of them, Proteus mirabilis, P. vulgaris, and P. penneri, cause human
20 disease.

The most common type of proteus infection involves the urinary tract, but septicemia, pneumonia and wound infections also occur. Proteus mirabilis is the species most often isolated and may account for up to 10% of all
25 acute, uncomplicated urinary tract infections. Species, rather than genus level identifica-tion of the causative organism is desirable because of differen-tial antibiotic susceptibility among the species.

The current method for identifying Proteus mirabilis from patient samples involves culture of the specimen on agar plates for 18-24 hours, followed by a battery of biochemical tests. The oligonucleotide sequence described below, when used as a probe in a nucleic acid hybridization assay, accurately identifies
30 Proteus mirabilis. The present invention reduces the number of tests which must be run on a sample, the time to identification and therefore, diagnosis and
35

treatment. This represents a significant improvement over prior art methods.

The probe specific for Proteus mirabilis was obtained with a primer complementary to a conserved region of 23S rRNA with the sequence 5'-CAG TCA GGA GTA TTT AGC CTT-3'.

The following sequence was characterized and shown to be specific for P. mirabilis. The phylogenetically nearest neighbors Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris and Salmonella enteritidis were used as comparisons with the sequence of Proteus mirabilis.

1. CCG TTC TCC TGA CAC TGC TAT TGA TTA AGA CTC

This probe is capable of hybridizing to the RNA of P. mirabilis in the region corresponding to base 270-305 of E. coli 23s rRNA. The probe is 33 bases in length and has a Tm of 66°C. To demonstrate the reactivity and specificity of the probe for P. mirabilis, it was used in a hybridization assay. ³²P-end-labeled oligonucleotide probe was mixed with RNA released from at least 10⁷ organisms in 1% sodium dodecyl sulfate, 0.48 M sodium phosphate, pH 6.8, 1 mM EDTA, 1 mM EGTA (0.2 ml final volume) and incubated at 64°C for 2 hours. Following incubation, 5 ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added and the mixture incubated at 64°C for 10 minutes. The sample was centrifuged and the supernatant removed. Five ml of wash solution (0.12 M sodium phosphate, pH 6.8, 0.02% sodium dodecyl sulfate) was added, the sample vortexed, centrifuged and the supernatant was removed. The amount of radioactivity bound to the hydroxyapatite was determined by scintillation counting. The results are shown in Table 48 and demonstrate that the probe reacts well with P. mirabilis and does not react with 27 other closely

related bacteria. Table 49 shows that the probe does not react with 24 other phylogenetically diverse bacteria and two yeasts tested in the same manner as the organisms in Table 48.

5

TABLE 48

10 HYBRIDIZATION OF PROTEUS MIRABILIS PROBE
TO CLOSELY RELATED ORGANISMS

	<u>Organism Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Citrobacter diversus	27156	1.1
	Citrobacter freundii	8090	1.1
15	Citrobacter freundii	6750	1.0
	Enterobacter aerogenes	13048	1.0
	Enterobacter agglomerans	27155	1.0
	Enterobacter cloacae	e13047	1.1
	Enterobacter gergoviae	33028	1.0
20	Enterobacter sakazakii	29544	1.1
	Escherichia coli	10798	1.2
	Escherichia coli	11775	1.2
	Escherichia coli	29417	1.2
	Klebsiella oxytoca	13182	1.0
25	Klebsiella ozaenae	11296	1.1
	Klebsiella planticola	33531	0.9
	Klebsiella pneumoniae	13883	1.3
	Klebsiella pneumoniae	23357	1.1
	Klebsiella		
30	rhinoscleromatis	13884	1.2
	Klebsiella terrigena	33257	1.1
	Klebsiella trevisanii	33558	1.0
	Kluyvera ascorbata	33433	0.9
	Proteus mirabilis	25933	69.0
35	Proteus penneri	33519	2.5
	Proteus vulgaris	13315	1.7
	Providencia		
	alcalifaciens	9886	1.1
	Providencia rettgeri	29944	1.3
40	Providencia stuartii	29914	1.1
	Salmonella arizonae	29933	1.1
	Salmonella enteritidis	13076	0.8

45

SUBSTITUTE SHEET

TABLE 49

HYBRIDIZATION OF PROTEUS MIRABILIS PROBE TO
PHYLOGENETICALLY DIVERSE ORGANISMS

5	<u>Organism Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter calcoaceticus	33604	0.8
	Bacillus subtilis	6051	1.2
	Bacteroides fragilis	23745	0.9
	Branhamella catarrhalis	25238	0.7
10	Campylobacter jejuni	33560	1.0
	Candida krusei	34135	0.8
	Chromobacterium violaceum	29094	1.1
	Clostridium perfringens	13124	0.9
	Deinococcus radiodurans	35073	0.8
15	Derxia gummosa	15994	0.8
	Hafnia alvei	13337	0.9
	Morganella morganii	25830	0.9
	Pseudomonas aeruginosa	10145	1.0
	Pseudomonas cepacia	17762	0.9
20	Rahnella aquatilis	33071	0.9
	Rhodospirillum rubrum	11170	0.8
	Serratia marcescens	13880	0.9
	Serratia odorifera	33077	0.9
	Staphylococcus aureus	e12600	0.8
25	Staphylococcus epidermidis	14990	0.8
	Streptococcus mitis	9811	0.8
	Streptococcus pneumoniae	e6306	0.9
	Torulopsis glabrata	2001	0.9
	Vibrio parahaemolyticus	17802	0.8
30	Xanthomonas maltophilia	13637	1.1
	Yersinia enterocolitica	9610	0.8

Example 17

35 Members of the genus Salmonella are motile, gram negative, non-sporeforming bacilli which belong in the family Enterobacteriaceae. All salmonellae are highly related and some microbiologists consider them to be one species. Five subgroups have been identified using

40 nucleic acid homology studies and over 1400 different serotypes have been described. All serotypes have been implicated in human enteric disease ranging from self-

limited gastroenteritis with mild symptoms, to severe gastroenteritis with bacteremia, to typhoid fever, a potentially life-threatening illness. S. choleraesuis, S. paratyphi A and S. typhi are the serotypes most often associated with severe disease and bacteremia. Diagnosis of Salmonella-induced enteritis is dependent upon detection of the organism in stool samples. Because infection occurs primarily by ingestion of contaminated milk, food and water, methods for identifying Salmonella in these products before release to consumers is critical.

Current methods for detection of members of the genus Salmonella involve culture of the specimen for 1-3 days on selective media followed by a battery of biochemical tests. Often an enrichment step is needed to isolate Salmonella from clinical samples or food products. The oligonucleotide sequences shown below, when used in a hybridization assay, accurately identify members of the genus Salmonella. The present inventive probes are specific for all members of the genus and do not react with the other closely related Enterobacteriaceae genera. These inventive probes reduce the number of tests which must be run on a sample and greatly reduce the time to identification. This represents a significant improvement over prior art methods.

The probes specific for the genus Salmonella were obtained with two primers complementary to 16S and 23S rRNA. Sequence 1 was obtained using a 16S primer with the sequence 5' TTA CTA GCG ATT CCG ACT TCA 3'. Sequence 2 was obtained using a 23S primer with the sequence 5' CAG TCA GGA GTA TTT AGC CTT 3'. The following sequences were characterized and shown to be specific for the genus Salmonella:

1. CTC CTT TGA GTT CCC GAC CTA ATC GCT GGC

2. CTC ATC GAG CTC ACA GCA CAT GCG CTT TTG TGT A
Sequence 1, from 16S rRNA, is 30 bases in length and has a T_m of 73°C. Sequence 2, from 23S rRNA, is 34 bases long and has a T_m of 71°C. These probes are capable of hybridizing in the regions corresponding to bases 1125-1155 of E. coli 16s rRNA and 335-375 of E. coli 23s rRNA, respectively. To demonstrate the reactivity and specificity of probe 1 for members of the genus Salmonella, ^{32}P -end-labeled oligonucleotide was tested as a probe in a hybridization reaction. Purified RNA, or RNA released from at least 10^7 organisms by standard methods, was mixed with 1 ml hybridization buffer (final concentration 43% diisobutyl sulfosuccinate, 60mM sodium phosphate pH 6.8, 1mM EDTA, 1mM EGTA) and incubated at 72°C for 2-12 hours. Following incubation, 5 ml of separation solution (2% hydroxyapatite, 0.12 M sodium phosphate, pH 6.8, 0.02% sodium dodecyl sulfate) was added and the sample were mixed, incubated at 72°C for 5 minutes, centrifuged and the supernatants removed. Four ml of wash solution (0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate) was added and the samples were vortexed, centrifuged, and the supernatants removed. The amount of radioactivity bound to the hydroxyapatite was determined by scintillation counting. The results shown in Table 50 indicate that a combination of the two probes hybridized to the 5 subgroups of Salmonella and to all 31 of the serotypes which were tested.

TABLE 50

5 HYBRIDIZATION OF SALMONELLA PROBES 1 AND 2
TO MEMBERS OF THE GENUS SALMONELLA

		% Probe Bound		
<u>Subgroup and Organism</u>		<u>ATCC#</u>	<u>Probe 1</u>	<u>Probe 2</u>
10	I <i>Salmonella choleraesuis</i>	10708	24	40
	I <i>Salmonella enteritidis</i>	13076	15	67
	I <i>Salmonella paratyphi</i> A	9150	1.4	70
	I <i>Salmonella</i> sp. serotype anatum	9270	40	26
15	I <i>Salmonella</i> sp. serotype cubana	12007	54	35
	I <i>Salmonella</i> sp. serotype give	9268	12	40
	I <i>Salmonella</i> sp. serotype heidelberg	8326	53	33
20	I <i>Salmonella</i> sp. serotype illinois	11646	36	46
	I <i>Salmonella</i> sp. serotype montevideo	8387	35	32
	I <i>Salmonella</i> sp. serotype newington	29628	52	34
25	I <i>Salmonella</i> sp. serotype newport	6962	3.4	36
	I <i>Salmonella</i> sp. serotype putten	15787	34	39
	30	I <i>Salmonella</i> sp. serotype saintpaul	9712	28
I <i>Salmonella</i> sp. serotype senftenberg		8400	38	43
I <i>Salmonella</i> sp. serotype simsbury		12004	29	29
35	I <i>Salmonella</i> sp. serotype sloterdijk	15791	34	30
	I <i>Salmonella</i> sp. serotype thompson	8391	32	41
	40	I <i>Salmonella</i> sp. serotype vellore	15611	35
I <i>Salmonella typhi</i>		19430	7.0	21
I <i>Salmonella typhimurium</i>		14028	69	69
45	II <i>Salmonella salamae</i>	6959	3.0	46
	II <i>Salmonella</i> sp. serotype maarssen	15793	6.6	30
	III <i>Salmonella arizonae</i>	33952	2.9	38
	III <i>Salmonella arizonae</i>	12324	5.5	42
	III <i>Salmonella arizonae</i>	29933	2.3	62

SUBSTITUTE SHEET

	III	Salmonella arizonae	29934	63	12
	III	Salmonella arizonae	12323	4.0	39
	III	Salmonella arizonae	12325	51	1.9
	IV	Salmonella sp.	15783	5.8	8.0
5		serotype harmelen			
	IV	Salmonella sp.	29932	7.5	40
		serotype ochsenzoll			
	V	Salmonella sp.	cdc1319	60	1.8
		serotype bongor			
10					

The specificity of the probes for members of the genus Salmonella was demonstrated with hybridization reactions containing RNA from organisms closely related to Salmonella. The results are shown in Table 51.

TABLE 51

HYBRIDIZATION OF SALMONELLA PROBES 1 AND 2
TO RNA OF CLOSELY RELATED ORGANISMS

	Organism	ATCC#	% Probe Bound	
			Probe 1	Probe 2
25	Citrobacter freundii	6750	2.2	0
	Edwardsiella tarda	15947	0	0
	Enterobacter agglomerans	27155	0.6	0
	Enterobacter cloacae	13047	0	0
	Enterobacter sakazakii	29544	0	0
30	Escherichia coli	10798	0	0
	Escherichia coli	29417	0	0
	Klebsiella pneumoniae	23357	0.7	0
	Kluyvera ascorbata	33433	0	0.5
	Proteus mirabilis	25933	0.2	0
35	Shigella flexneri	29903	0	0

* % Probe Bound = counts bound to hydroxyapatite-
counts bound when no RNA present/total counts used in
assay

40

Table 52 shows that Salmonella probes 1 and 2 do not hybridize to phylogenetically diverse organisms.

SUBSTITUTE SHEET

TABLE 52

HYBRIDIZATION OF SALMONELLA PROBES 1 AND 2 TO
RNA OF A PHYLOGENETIC CROSS SECTION OF ORGANISMS

5	Organism	ATCC#	% Probe Bound*	
			Probe 1	Probe 2
	Acinetobacter calcoaceticus	33604	1.1	0.1
	Bacillus subtilis	6051	0	0.5
	Bacteroides fragilis	23745	0.1	0
10	Branhamella catarrhalis	25238	0.9	0
	Campylobacter jejuni	33560	0	0.2
	Candida krusei	34135	0.4	0.3
	Chromobacterium violaceum	29094	1.7	0
	Clostridium perfringens	13124	0.3	0
15	Deinococcus radiodurans	35073	1.6	0.1
	Derxia gummosa	15994	1.2	0
	Hafnia alvei	13337	1.8	0
	Morganelli morganii	25830	0	1.1
	Pseudomonas aeruginosa	10145	0.5	0.7
20	Pseudomonas cepacia	17762	0	0
	Pseudomonas maltophilia	13637	1.9	0
	Rahnella aquatilis	33071	1.2	0.3
	Rhodospirillum rubrum	11170	0.9	0
	Serratia marcescens	13880	0	0
25	Serratia odorifera	33077	2.6	0.2
	Staphylococcus aureus	e12600	0.2	0
	Staphylococcus epidermidis	14990	0	0
	Streptococcus mitis	9811	1.2	0.7
	Streptococcus pneumoniae	e6306	0	0
30	Torulopsis glabrata	2001	0	0
	Vibrio parahaemolyticus	17802	0	0.2
	Yersinia enterocolitica	9610	0	0

35 *% Probe Bound = Counts bound to hydroxyapatite-
counts bound when no RNA present/total counts used in
assay

Example 18

40 Escherichia coli is a gram negative,
nonsporeforming bacillus which belongs in the family
Enterobacteriaceae. Five species of Escherichia have
been described: E. coli, which accounts for >99% of the

clinical isolates, E. hermannii, E. blattae, E. vulneris and E. fergusonii. E. coli is a leading cause of urinary tract infections, bacteremia and neonatal meningitidis, and can cause a type of gastroenteritis known as traveller's diarrhea.

The current method for identifying E. coli from patient samples involves culture of the specimen on agar plates for 18-72 hours, followed by a battery of biochemical tests on isolated colonies. The oligonucleotide sequence described below, when used as a probe in a nucleic acid hybridization assay, accurately detects E. coli even in the presence of other organisms. The present invention reduces the number of tests which must be run on a sample and reduces the time to identification and therefore diagnosis and treatment. This represents a significant improvement over prior art methods.

The probe specific for E. coli was derived from the published E. coli sequence (Brosius, et al. Proc. Natl. Acad. Sci. U.S.A. 75:4801-4805 (1978)), using Proteus vulgaris (Carbon, et al., Nuc. Acids Res. 9:2325-2333 (1981)), Klebsiella pneumoniae, Salmonella enteritidis, Enterobacter gergoviae and Citrobacter freundii for comparison. The probe sequence is shown below.

1. GCA CAT TCT CAT CTC TGA AAA CTT CCG TGG

It hybridizes to RNA of E. coli in the region of 995-1030 of 16s rRNA. The probe is 30 bases in length and has a T_m of 66°C. To demonstrate the reactivity and specificity of the probe for E. coli, it was used in a hybridization assay. 32 P-end-labeled oligonucleotide probe was mixed with two unlabeled oligonucleotides of sequence 5'-TGG ATG TCA AGA CCA GGT AAG GTT CTT CGC GTT GCA TCG-3' and 5'-CTG ACG ACA GCC ATG CAG CAC CTG TCT CAC GGT TCC CGA AGG CA-3' and with purified RNA, or RNA released from cells with detergent and heat, in 1%

sodium dodecyl sulfate (SDS), 0.48 M sodium phosphate pH 6.8, 1mM EDTA, 1 mM EGTA (0.2 ml final volume) and incubated at 60°C for 2 hours. Following incubation, 5 ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added and the mixture incubated at 60°C for 10 minutes. The sample was centrifuged and the supernatant removed. Five ml of wash solution (0.12 M sodium phosphate, pH 6.8, 0.02% sodium dodecyl sulfate) was added, the sample vortexed, centrifuged and the supernatant was removed. The amount of radioactivity bound to the hydroxyapatite was determined by scintillation counting.

An example of a use for this probe would be to detect E. coli in urine samples. Table 53 shows that the probe detects 7 out of 8 strains of E. coli tested. The probe also reacts with E. fergusonii, an organism which would only rarely be found in urine.

Table 54 shows that the probe does not react with any other genus tested except Shigella, another organism rarely isolated from urine. These results show that the probe will be useful in detecting E. coli from urine samples.

25

TABLE 53

HYBRIDIZATION OF E. COLI TO ESCHERICHIA SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
30	Escherichia coli	10798	70
	E. coli	11775	67
	E. coli	23722	58
	E. coli	25404	68
	E. coli	25922	55
35	E. coli	29417	72
	E. coli	33780	0.8
	E. coli	35150	45

SUBSTITUTE SHEET

E. fergusonii	35469	55
E. hermanii	33650	0.7
E. vulneris	33821	0.8

5

TABLE 54

HYBRIDIZATION OF THE E. COLI PROBE TO
CLOSELY RELATED ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
10	Citrobacter freundii	6750	0.8
	Citrobacter freundii	8090	0.9
	Citrobacter freundii	29221	0.6
	Citrobacter freundii	33128	0.6
	Enterobacter aerogens	13048	1.2
15	Enterobacter agglomeans	27155	0.9
	Enterobacter cloacae	13047	0.9
	Enterobacter gergoviae	33023	0.7
	Enterobacter sakazakii	29544	0.6
	Klebsiella oxytoca	13182	0.7
20	Klebsiella pneumoniae	13883	0.7
	Proteus mirabilis	29906	0.7
	Proteus vulgaris	13315	0.8
	Shibella boydii	8700	76
	Shigella dysenteriae	13313	0.8
25	Shigella flexneri	29903	71
	Shigella sonnei	29930	75

30

Example 19

The bacteria encompass a morphologically and physiologically diverse group of unicellular organisms which occupy most natural environments. Although many bacteria are harmless or beneficial to their environment or host, some are harmful and cause disease. The presence of any bacteria in some locations is undesirable or indicative of disease (e.g., culture media, pharmaceutical products, body fluids such as blood, urine or cerebrospinal fluid, and tissue biopsies). Low levels of bacteria are considered acceptable in other products such as drinking water and

40

food products. Accordingly, there is a need for a means for detecting and quantitating bacteria in a sample.

The current method of detection and quantitation of total bacteria in a sample requires culture on multiple types of media under different conditions of temperature and atmosphere. To date, no single test exists to detect or quantitate all bacteria. The oligonucleotide sequences shown below, when used in a hybridization assay, detect a broad phylogenetic cross section of bacteria. The present invention reduces the number of tests which need to be performed and also reduces the time required for the assay. Comparison of the hybridization results from an unknown sample to a set of standards will allow some quantitation of the number of bacteria present. This represents a significant improvement over prior art methods.

The bacterial probes were designed following examination of published sequences of rRNA and sequences determined at Gen-Probe. The sequences used for the comparison include Agrobacterium tumefaciens (Yang et al., Proc. Natl. Acad. Sci. U.S.A., 82:4443, (1985), Anacystis nidulans (Tomiooka and Sugiura. Mol. Gen. Genet. 191:46, (1983); Douglas and Doolittle Nuc. Acids Res. 12:3373, (1984), Bacillus subtilis (Green et al., Gene 37:261. (1985), Bacillus stearothermophilus (Kop et al., DNA 3:347, (1984), Bacteroides fragilis (Weisburg et al., J. Bacteriol. 164:230, (1985), Chlamydia psittaci (Weisburg et al., J. Bacteriol. 167:570. (1986)), Desulfovibrio desulfuricans (Oyaizu and Woese, System. Appl. Microbiol. 6:257, (1985); Escherichia coli, (Brosius et al., Proc. Natl. Acad. Sci. U.S.A. 77:201, (1980); Flavobacterium heparinum (Weisburg et al., J. Bacteriol. 164:230, (1985); Heliobacterium chlorum (Woese et al., Science 229:762, (1985); Mycoplasma PG50 (Frydenberg and Christiansen, DNA 4:127,

(1985); Proteus vulgaris (Carbon et al., Nuc. Acids Res. 9:2325, (1981); Pseudomonas testosteroni (Yang et al., Proc. Natl. Acad. Sci. U.S.A. 82:4443, (1985); Rochalimaea quintana (Weisburg et al., Science 230:556, (1985); Saccharomyces cerevisiae (Rubstov et al., Nuc. Acids Res. 8:5779, (1980); Georgiev et al., Nuc. Acids Res. 9:6953, (1981); and human (Torczynski et al., DNA 4:283, (1985); Gonzalez et al., Proc. Natl. Acad. Sci. U.S.A. 82:7666, (1985)).

10 The following sequences were shown to hybridize to a broad phylogenetic cross section of bacteria and not to yeast or human rRNA:

1. CCA CTG CTG CCT CCC GTA GGA GTC TGG GCC
2. CCA GAT CTC TAC GCA TTT CAC CGC TAC ACG TGG
- 15 3. GCT CGT TGC GGG ACT TAA CCC AAC AT
4. GGG GTT CTT TTC GCC TTT CCC TCA CGG
5. GGC TGC TTC TAA GCC AAC ATC CTG
6. GGA CCG TTA TAG TTA CGG CCG CC
7. GGT CGG AAC TTA CCC GAC AAG GAA TTT CGC TAC C

20 Probe 1 is 30 bases long and has a T_m of 70°C. Probe 2 is 33 bases long and has a T_m of 69°C. Probe 3 is 26 bases long and has a T_m of 67°C. Probe 4 is 27 bases long and has a T_m of 69°C. Probe 5 is 24 bases long and has a T_m of 66°C. Probe 6 is 23 bases long and has a T_m of 62°C. Probe 7 is 34 bases long and has a T_m of 66°C. Probes 1-3 hybridize to 16S rRNA in the following regions, respectively, (corresponding to E. coli bases) 330-365; 675-715; and 1080-1110. Probes 4-7 hybridize to 23S rRNA in the following regions, respectively, (corresponding to E. coli bases) 460-490; 1050-1080; and 1900-1960 (probes 6 and 7). The oligonucleotides interact with regions on the rRNA which are highly conserved among eubacteria. This means that they can be used as bacterial probes in a hybridization assay. A second use is as a tool to obtain rRNA

sequence. For example, an oligonucleotide can be hybridized to the rRNA of interest and extended with reverse transcriptase. The sequence of the resulting DNA can be determined and used to deduce the complementary rRNA sequence as described in the Detailed Description of the Invention.

One application of the invention is to detect bacteria in urine (bacteriuria). To demonstrate the reactivity and specificity of the probes for bacteria found in urine, they were used in hybridization assays. ³²P-end-labeled or ¹²⁵I-labeled oligonucleotide probes were mixed with RNA released from cells by standard methods (e.g, the sonic disruption techniques described in Murphy et al., U.S. Patent App. Ser. No. 841,860, detergent with glass beads, or enzymatic lysis). Probe was mixed with RNA in 0.48 M sodium phosphate, pH 6.8, 1 mM EDTA, 1 mM EGTA, 1% sodium dodecyl sulfate (0.2 ml final volume) and hybridized at 60°C for 2 hours. Five ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added and the mixture incubated at 60°C for 10 minutes. The mixture was centrifuged and the supernatant removed. Five ml of wash solution (0.12 M sodium phosphate, pH 6.8, 0.02% sodium dodecyl sulfate) was added and the sample was mixed, centrifuged and the supernatant removed. The amount of radioactivity bound to the hydroxyapatite was determined by scintillation counting. Tables 55-68 demonstrate the specificity of these probes and show that a combination of probes could be used to detect all bacteria which have been tested.

Table 55 shows that probe 1 hybridizes to the RNA of bacteria commonly isolated from urine and does not detect yeast RNA. Table 56 shows that probe 1 detects phylogenetically diverse bacteria and does not hybridize to human RNA.

TABLE 55

5 HYBRIDIZATION OF BACTERIAL PROBE 1
TO RNA OF ORGANISMS FOUND IN URINE

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe*</u> <u>Bound</u>
	Candida albicans	18804	2.6
10	Candida krusei	34135	2.2
	Candida parapsilosis	22019	2.9
	Candida tropicalis	750	2.5
	Citrobacter freundii	8090	69
	Enterobacter aerogens	13048	70
15	Enterobacter cloacae	13047	71
	Escherichia coli	11775	67
	Klebsiella oxytoca	13182	70
	Klebsiella pneumoniae	13883	72
	Morganella morganii	25830	66
20	Proteus mirabilis	29906	71
	Proteus vulgaris	13315	67
	Providencia stuartii	29914	69
	Pseudomonas aeruginosa	10145	76
	Pseudomonas fluorescens	13525	73
25	Serratia marcescens	13880	66
	Staphylococcus aureus	12600	57
	Staphylococcus epidermidis	14990	68
	Streptococcus agalactiae	13813	68
30	Streptococcus faecalis	19433	51
	Streptococcus faecium	19434	53
	Torulopsis glabrata	2001	2.3
	Ureaplasma urealyticum	27618	54

TABLE 56

5 HYBRIDIZATION OF BACTERIAL PROBE 1
TO RNAs OF A CROSS SECTION OF PHYLOGENETICALLY
DIVERSE ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe*</u> <u>Bound</u>
10	Acinetobacter calcoaceticus	23055	65
	Bacillus subtilis	6051	73
	Bacteroides fragilis	23745	61
	Branhamella catarrhalis	25238	72
	Campylobacter jejuni	33560	64
15	Chlamydia trachomatis	VR878	14
	Chromobacterium violaceum	29094	71
	Clostridium perfringens	13124	74
	Corynebacterium xerosis	373	38
	Deinococcus radiodurans	35073	47
20	Derxia gummosa	15994	65
	Gardnerella vaginalis	14018	67
	Hafnia alvei	13337	60
	Lactobacillus acidophilus	4356	56
	Moraxella osloensis	19976	61
25	Mycobacterium smegmatis	14468	47
	Mycoplasma hominis	14027	58
	Neisseria gonorrhoeae	19424	58
	Rahnella aquatilis	33071	74
	Rhodospirillum rubrum	11170	73
30	Vibrio parahaemolyticus	17802	75
	Human		2.5

35 Table 57 shows that Probe 2 hybridizes to the RNA of
bacteria commonly found in urine except Ureaplasma
urealyicum and does not hybridize to yeast rRNA..

TABLE 57

40 HYBRIDIZATION OF BACTERIAL PROBE 2
TO RNA OF ORGANISMS FOUND IN URINE

<u>Organism</u>	<u>ATCC#</u>	<u>%Probe*</u> <u>Bound</u>
Candida albicans	18804	2.5

109

	Candida krusei	34135	1.8
	Candida parapsilosis	22019	1.6
	Candida tropicalis	750	1.4
	Citrobacter freundii	8090	61
5	Enterobacter aerogenes	13048	57
	Enterobacter cloacae	13047	61
	Escherichia coli	11775	67
	Klebsiella oxytoca	13182	67
	Klebsiella pneumoniae	13883	51
10	Morganella morganii	25830	69
	Proteus mirabilis	29906	69
	Proteus vulgaris	13315	69
	Providencia stuartii	29914	66
	Pseudomonas aeruginosa	10145	59
15	Pseudomonas fluorescens	13525	58
	Serratia marcescens	13880	64
	Staphylococcus aureus	12600	60
	Staphylococcus epidermidis	14990	60
20	Streptococcus agalactiae	13813	54
	Streptococcus faecalis	19433	37
	Streptococcus faecium	19434	58
	Torulopsis glabrata	2001	1.5
	Ureaplasma urealyticum	27618	3.2
25			

Table 58 shows that probe 2 detects phylogenetically
 30 diverse bacteria and does not hybridize to human rRNA.

TABLE 58

35 HYBRIDIZATION OF BACTERIAL PROBE 2 TO RNAs
 OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe*</u> <u>Bound</u>
	Acinetobacter calcoaceticus	23055	76
40	Bacillus subtilis	6051	75
	Bacteroides fragilis	23745	2.0
	Branhamella catarrhalis	25238	70
	Campylobacter jejuni	33560	2.5
	Chlamydia trachomatis	VR878	16
45	Chromobacterium violaceum	29094	61

SUBSTITUTE SHEET

CGK00033244

110

	<i>Clostridium perfringens</i>	13124	66
	<i>Corynebacterium xerosis</i>	373	3.8
	<i>Deinococcus radiodurans</i>	35073	6.0
	<i>Derxia gummosa</i>	15994	61
5	<i>Gardnerella vaginalis</i>	14018	2.0
	<i>Hafnia alvei</i>	13337	72
	<i>Lactobacillus acidophilus</i>	4356	50
	<i>Moraxella osloensis</i>	19976	64
10	<i>Mycobacterium smegmatis</i>	14468	19
	<i>Mycoplasma hominis</i>	14027	34
	<i>Neisseria gonorrhoeae</i>	19424	71
	<i>Rahnella aquatilis</i>	33071	77
	<i>Rhodospirillum rubrum</i>	11170	1.5
15	<i>Vibrio parahaemolyticus</i>	17802	73
	<i>Yersinia enterocolitica</i>	9610	76
	Human		2.0

20

Table 59 shows that probe 3 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

25

TABLE 59

HYBRIDIZATION OF BACTERIAL PROBE 3 TO RNA OF ORGANISMS FOUND IN URINE

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe* Bound</u>
30	<i>Candida albicans</i>	18804	1.4
	<i>Candida krusei</i>	34135	1.5
	<i>Candida parapsilosis</i>	22019	2.2
	<i>Candida tropicalis</i>	750	2.6
35	<i>Citrobacter freundii</i>	8090	79
	<i>Enterobacter aerogenes</i>	13048	40
	<i>Enterobacter cloacae</i>	13047	44
	<i>Escherichia coli</i>	11775	67
	<i>Klebsiella oxytoca</i>	13182	38
40	<i>Klebsiella pneumoniae</i>	13883	45
	<i>Morganella morganii</i>	25830	57
	<i>Proteus mirabilis</i>	29906	40
	<i>Proteus vulgaris</i>	13315	51
	<i>Providencia stuartii</i>	29914	54
45	<i>Pseudomonas aeruginosa</i>	10145	61
	<i>Pseudomonas fluorescens</i>	13525	56

SUBSTITUTE SHEET

CGK00033245

111

	Serratia marcescens	13880	54
	Staphylococcus aureus	12600	37
	Staphylococcus		
	epidermidis	14990	20
5	Streptococcus agalactiae	13813	34
	Streptococcus faecalis	19433	20
	Streptococcus faecium	19434	47
	Torulopsis glabrata	2001	1.9
	Ureaplasma urealyticum	27618	26

10

Table 60 shows that probe 3 detects phylogenetically diverse bacteria and does not hybridize to human rRNA.

15

TABLE 60

HYBRIDIZATION OF BACTERIAL PROBE 3 TO RNAs
OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE ORGANISMS

20	<u>Organism Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter		
	calcoaceticus	23055	69
	Bacillus subtilis	6051	35
25	Bacteroides fragilis	23745	1.2
	Branhamella catarrhalis	25238	43
	Campylobacter jejuni	33560	55
	Chlamydia trachomatis	VR878	42
	Chromobacterium		
30	violaceum	29094	69
	Clostridium perfringens	13124	62
	Corynebacterium xerosis	373	23
	Deinococcus radiodurans	35073	30
	Derxia gummosa	15994	67
35	Gardnerella vaginalis	14018	40
	Hafnia alvei	13337	56
	Lactobacillus		
	acidophilus	4356	36
	Moraxella osloensis	19976	64
40	Mycobacterium smegmatis	14468	77
	Mycoplasma hominis	14027	1.5
	Neisseria gonorrhoeae	19424	26
	Rahnella aquatilis	33071	66
	Rhodospirillum rubrum	11170	51
45	Vibrio parahaemolyticus	17802	68
	Yersinia enterocolitica	9610	68
	Human		0.9

SUBSTITUTE SHEET

CGK00033246

Table 61 shows that probe 4 hybridizes to the RNA of
 5 bacteria commonly found in urine and does not detect
 yeast rRNA.

TABLE 61

10 HYBRIDIZATION OF BACTERIAL PROBE 4 TO RNA OF
 ORGANISMS FOUND IN URINE.

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Candida albicans	18804	4.5
15	Candida krusei	34135	2.5
	Candida parapsilosis	22019	2.7
	Candida tropicalis	750	2.5
	Citrobacter freundii	8090	55
	Enterobacter aerogenes	13048	52
20	Enterobacter cloacae	13047	57
	Escherichia coli	11775	70
	Klebsiella oxytoca	13182	70
	Klebsiella pneumoniae	13883	43
	Morganella morganii	25830	74
25	Proteus mirabilis	29906	74
	Proteus vulgaris	13315	73
	Providencia stuartii	29914	73
	Pseudomonas aeruginosa	10145	76
	Pseudomonas fluorescens	13525	79
30	Serratia marcescens	13880	74
	Staphylococcus aureus	12600	73
	Staphylococcus epidermidis	14990	73
	Streptococcus agalactiae	13813	70
35	Streptococcus faecalis	19433	37
	Streptococcus faecium	19434	63
	Torulopsis glabrata	2001	2.2
	Ureaplasma urealyticum	27618	43

40

Table 62 shows that probe 4 detects phylogenetically
 diverse bacteria and does not hybridize to human rRNA.

SUBSTITUTE SHEET

TABLE 62

HYBRIDIZATION OF BACTERIAL PROBE 4 TO RNAs
OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE
ORGANISMS

	<u>Organism Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter		
10	calcoaceticus	23055	69
	Bacillus subtilis	6051	55
	Bacteroides fragilis	23745	3.0
	Branhamella catarrhalis	25238	59
	Campylobacter jejuni	33560	65
15	Chlamydia trachomatis	VR878	50
	Chromobacterium		
	violaceum	29094	61
	Clostridium perfringens	13124	57
	Corynebacterium xerosis	373	9.5
20	Deinococcus radiodurans	35073	63
	Derxia gummosa	15994	65
	Gardnerella vaginalis	14018	57
	Hafnia alvei	13337	67
	Lactobacillus		
25	acidophilus	4356	68
	Moraxella osloensis	19976	68
	Mycobacterium smegmatis	14468	28
	Mycoplasma hominis	14027	74
	Neisseria gonorrhoeae	19424	76
30	Rahnella aquatilis	33071	68
	Rhodospirillum rubrum	11170	59
	Vibrio parahaemolyticus	17802	75
	Yersinia enterocolitica	9610	74
35	Human		2.8

Table 63 shows that probe 5 hybridizes to the RNA of
bacteria commonly found in urine and does not detect
yeast rRNA.

TABLE 63

HYBRIDIZATION OF BACTERIAL PROBE 5 TO RNA OF
ORGANISMS FOUND IN URINE

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Candida albicans	18804	1.8
	Candida krusei	34135	1.7
10	Candida parapsilosis	22019	2.2
	Candida tropicalis	750	1.8
	Citrobacter freundii	8090	39
	Enterobacter aerogenes	13048	38
	Enterobacter cloacae	13047	43
15	Escherichia coli	11775	31
	Klebsiella oxytoca	13182	38
	Klebsiella pneumoniae	13883	66
	Morganella morganii	25830	50
	Proteus mirabilis	29906	44
20	Proteus vulgaris	13315	52
	Providencia stuartii	29914	44
	Pseudomonas aeruginosa	10145	47
	Pseudomonas fluorescens	13525	25
	Serratia marcescens	13880	35
25	Staphylococcus aureus	12600	26
	Staphylococcus epidermidis	14990	37
	Streptococcus agalactiae	13813	29
	Streptococcus faecalis	19433	14
30	Streptococcus faecium	19434	33
	Torulopsis glabrata	2001	2.2
	Ureaplasma urealyticum	27618	73

35 Table 64 shows that probe 5 detects phylogenetically diverse bacteria and does not hybridize to human RNA.

TABLE 64

5 HYBRIDIZATION OF BACTERIAL PROBE 5 TO RNAs
OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE
ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter		
10	calcoaceticus	23055	20
	Bacillus subtilis	6051	53
	Bacteroides fragilis	23745	44
	Branhamella catarrhalis	25238	22
	Campylobacter jejuni	33560	35
15	Chromobacterium		
	violaceum	29094	59
	Clostridium perfringens	13124	63
	Corynebacterium xerosis	373	1.7
	Deinococcus radiodurans	35073	5.7
20	Derxia gummosa	15994	14
	Gardnerella vaginalis	14018	1.6
	Hafnia alvei	13337	44
	Lactobacillus		
	acidophilus	4356	1.5
25	Moraxella osloensis	19976	7.2
	Mycobacterium smegmatis	14468	39
	Mycoplasma hominis	14027	21
	Neisseria gonorrhoeae	19424	40
	Rahnella aquatilis	33071	55
30	Rhodospirillum rubrum	11170	17
	Vibrio parahaemolyticus	17802	66
	Yersinia enterocolitica	9610	64
	Human		1.6

35

Table 65 shows that probe 6 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

TABLE 65

5 HYBRIDIZATION OF BACTERIAL PROBE 6 TO RNA OF
ORGANISMS FOUND IN URINE

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Candida albicans	18804	3.0
10	Candida krusei	34135	2.0
	Candida parapsilosis	22019	2.2
	Citrobacter freundii	8090	54
	Enterobacter aerogenes	13048	50
	Enterobacter cloacae	13047	58
15	Escherichia coli	11775	63
	Klebsiella oxytoca	13182	54
	Klebsiella pneumoniae	13883	55
	Morganella morganii	25830	60
	Proteus mirabilis	29906	64
20	Proteus vulgaris	13315	67
	Providencia stuartii	29914	64
	Pseudomonas aeruginosa	10145	65
	Pseudomonas fluorescens	13525	31
	Serratia marcescens	13880	67
25	Staphylococcus aureus	12600	53
	Staphylococcus epidermidis	14990	34
	Streptococcus agalactiae	13813	31
	Streptococcus faecium	19434	18
30	Torulopsis glabrata	2001	2.5

Table 66 shows that probe 6 detects some
35 phylogenetically diverse bacteria and does not hybridize
to human rRNA.

TABLE 66

HYBRIDIZATION OF BACTERIAL PROBE 5 TO RNAs
OF A CROSS SECTION OF PHYLOGENETICALLY
DIVERSE ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter		
10	calcoaceticus	23055	73
	Bacteroides fragilis	23745	7.0
	Branhamella catarrhalis	25238	4.0
	Deinococcus radiodurans	35073	5.5
	Derxia gummosa	15994	3.0
15	Gardnerella vaginalis	14018	2.0
	Hafnia alvei	13337	3.5
	Lactobacillus		
	acidophilus	4356	17
	Moraxella osloensis	19976	62
20	Mycoplasma hominis	14027	44
	Rahnella aquatilis	33071	56
	Yersinia enterocolitica	9610	50
	Human		4.0

Table 67 shows that probe 7 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

TABLE 67

HYBRIDIZATION OF BACTERIAL PROBE 7 TO RNA
OF ORGANISMS FOUND IN URINE

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Candida albicans	18804	2.1
	Candida krusei	34135	2.0
40	Candida tropicalis	750	2.2
	Citrobacter freundii	8090	67
	Enterobacter aerogenes	13048	69
	Enterobacter cloacae	13047	78

118

	<i>Escherichia coli</i>	11775	75
	<i>Klebsiella oxytoca</i>	13882	79
	<i>Klebsiella pneumoniae</i>	13883	77
	<i>Morganella morganii</i>	25830	76
5	<i>Proteus mirabilis</i>	29906	77
	<i>Proteus vulgaris</i>	13315	79
	<i>Providencia stuartii</i>	29914	64
	<i>Pseudomonas aeruginosa</i>	10145	76
	<i>Pseudomonas fluorescens</i>	13525	78
10	<i>Serratia marcescens</i>	13880	66
	<i>Staphylococcus aureus</i>	12600	71
	<i>Staphylococcus epidermidis</i>	14990	75
	<i>Streptococcus agalactiae</i>	13813	70
15	<i>Streptococcus faecalis</i>	19433	58
	<i>Streptococcus faecium</i>	19434	68
	<i>Torulopsis glabrata</i>	2001	2.4
	<i>Ureaplasma urealyticum</i>	27618	21

Table 68 shows that probe 7 detects phylogenetically diverse bacteria and does not hybridize to human rRNA.

25

TABLE 68

HYBRIDIZATION OF BACTERIAL PROBE 7 TO RNAs
OF A CROSS SECTION OF PHYLOGENETICALLY
DIVERSE ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	<i>Acinetobacter calcoaceticus</i>	23055	86
	<i>Bacillus subtilis</i>	6051	83
	<i>Bacteroides fragilis</i>	23745	69
35	<i>Branhamella catarrhalis</i>	25238	74
	<i>Campylobacter jejuni</i>	33560	5.3
	<i>Chlamydia trachomatis</i>	VR878	41
	<i>Chromobacterium violaceum</i>	29094	69
40	<i>Clostridium perfringens</i>	13124	68
	<i>Corynebacterium xerosis</i>	373	23
	<i>Deinococcus radiodurans</i>	35073	70
	<i>Derxia gummosa</i>	15994	69
	<i>Gardnerella vaginalis</i>	14018	68
45	<i>Hafnia alvei</i>	13337	77

SUBSTITUTE SHEET

CGK00033253

	Moraxella osloensis	19976	68
	Mycobacterium smegmatis	14468	64
	Mycoplasma hominis	14027	4.0
	Neisseria gonorrhoeae	19424	53
5	Rahnella aquatilis	33071	72
	Rhodospirillum rubrum	11170	73
	Vibrio parahaemolyticus	17802	67
	Yersinia enterocolitica	9610	66
10	Human		2.2

Example 20

Fungi encompass a morphologically and physiologically diverse group of simple eucaryotic organisms. We estimate, using published sequences of three fungi, Neurospora crassa, Podospora, and Saccharomyces, that the rRNA of fungi are 58-60% homologous to E. coli and 84-90% homologous to one another. Some fungi grow as single cells (yeasts), others as multinuclear filaments (molds) and still others can grow as either single cells or multicellular filaments (dimorphic fungi). Although many fungi are harmless inhabitants of their environments, others are harmful and cause disease. The presence of any fungi in some locations is undesirable or indicative of disease (e.g., culture media, pharmaceutical products, body fluids such as blood, urine or cerebrospinal fluid, and tissue biopsies). Low levels of fungi are considered acceptable in other products such as drinking water and food products. This has created the need for a means of detecting and quantitating fungi in a sample.

The current methods for detecting and quantifying fungi involve microscopic examination of samples and culture on different media. Although most yeasts can be grown from clinical samples in a matter of days, some filamentous fungi take up to four weeks culture time, after which special staining procedures, biochemical analysis and antigen tests are performed. The

oligonucleotide sequences below, when used in a hybridization assay, detect the five yeasts most commonly isolated in the clinical setting, Candida albicans, Torulopsis glabrata, Candida tropicalis,
 5 Candida parapsilosis and Candida krusei. Five other fungi representing the Trichosporon, Blastomyces, Cryptococcus and Saccharomyces genera are also detected. The present invention allows one step detection of these organisms and, in relation to culture, reduces the time
 10 to identification or elimination of these fungi as the cause of an infection. This represents a significant improvement over prior art methods.

The four probes which hybridize to the organisms of interest were identified using 3 primers complementary
 15 to conserved regions on 18S or 28S rRNA. Sequence 1 was obtained using an 18S primer with the sequence 5'-AGA ATT TCA CCT CTG-3'. Sequence 2 was obtained using a 28S primer with the sequence 5'-CCT TCT CCC GAA GTT ACG G-3'. Sequences 3 and 4 were obtained with a 28S primer
 20 with the sequence 5'-TTC CGA CTT CCA TGG CCA CCG TCC-3'. The following sequences were characterized and shown to hybridize to fungal rRNA. The sequences of Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Escherichia coli and human rRNA were used for comparison
 25 with the sequences of interest.

1. CCC GAC CGT CCC TAT TAA TCA TTA CGA TGG
2. CGA CTT GGC ATG AAA ACT ATT CCT TCC TGT GG
3. GCT CTT CAT TCA ATT GTC CAC GTT CAA TTA AGC

AAC

30

AAG G

4. GCT CTG CAT TCA AAC GTC CGC GTT CAA TAA AGA

AAC

AGG G

Sequence 1, from 18S rRNA, is 30 bases in length
 35 and has a T_m of 68°C. Sequence 2, from 23S rRNA, is 32

SUBSTITUTE SHEET

bases in length and has a T_m of 67°C. Sequence 3, from 23S rRNA, is 40 bases in length and has a T_m of 66°C. Sequence 4, from 23S rRNA, is 40 bases in length and has a T_m of 68°C. Sequence 1 hybridizes in the region corresponding to position 845-880 of Saccharomyces cerevisiae 18s rRNA. Sequence 2 hybridizes in the region corresponding to position 1960-2000 of Saccharomyces cerevisiae 28s rRNA and sequences 3 and 4 hybridize in the region of 1225-1270 of the 28s rRNA.

To demonstrate the reactivity and specificity of these probes for fungal RNA, they were used in hybridization assays. ^{32}P - or ^{125}I -labeled oligonucleotide probes were mixed with purified RNA or RNA released from cells by standard lysis techniques in 0.2 ml of 0.48M sodium phosphate pH 6.8, 1% sodium dodecyl sulfate, 1mM EDTA, 1mM EGTA and incubated at 60°C for 2 hours. Following incubation, 5 ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added and the samples incubated 10 minutes at 60°C. The samples were centrifuged and the supernatants removed. Five ml of 0.12M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added, the samples were mixed, centrifuged and the supernatants removed. The results are shown in Table 69. Probe 1 detects all ten fungi which were tested, probe 2 detects all six of the yeasts which were tested, probe 3 detects five of the six yeasts, and probe 4 detects C. krusei only. Thus probe 4 could be used to detect and identify C. krusei in samples, probe 1, 2 or combination of 3 and 4 could be used to detect the yeasts, and probe 1 could be used to detect any of the ten organisms listed in Table 69.

One potential use for these probes is to identify yeasts in urine samples or other normally sterile body fluids. The probes were hybridized to a panel of

bacteria most commonly isolated from urine and shown not to react (Table 70). Table 71 shows that the probes do not hybridize to phylogenetically diverse bacteria or to human RNA.

5

TABLE 69

HYBRIDIZATION OF YEAST PROBES TO YEAST RNA

	<u>Organism</u>	<u>ATCC#</u>	% Probe Bound			
			#1	#2	#3	#4
	Blastomyces dermatitidis	C.I.	25	1.4	1.5	1.5
	Candida albicans	18804	40	63	56	2.0
	C. krusei	34135	73	62	2.2	70
15	C. parapsilosis	22019	71	63	65	2.0
	C. tropicalis	750	62	71	71	2.0
	Cryptococcus laurentii	C.I.	43	1.4	1.5	1.5
	Cryptococcus neoformans	C.I.	60	1.3	1.5	1.6
	Torulopsis glabrata	2001	61	44	62	2.0
20	Trichosporon beigelii	C.I.	57	1.3	2.1	1.5
	Saccharomyces cerevisiae	C.I.	41	67	53	1.9

C.I. = Clinical isolate

25

TABLE 70

HYBRIDIZATION OF FUNGAL PROBES 1-4 TO RNA
OF ORGANISMS FOUND IN URINE

	<u>Organism</u>	<u>ATCC#</u>	% Probe Bound			
			#1	#2	#3	#4
	Citrobacter freundii	8090	1.5	1.7	1.5	2.1
	Enterobacter aerogenes	13048	2.5	1.9	2.0	2.0
	Enterobacter cloacae	13047	2.5	1.6	2.6	2.0
	Escherichia coli	11775	3.0	2.0	1.6	1.5
35	Klebsiella oxytoca	13182	2.5	2.2	2.5	2.0
	Klebsiella pneumoniae	13883	2.5	2.2	2.1	2.0
	Morganella morganii	25830	2.0	2.8	1.7	1.9
	Proteus mirabilis	29906	2.5	1.9	2.3	2.0
	Proteus vulgaris	13315	2.0	2.2	2.0	1.5
40	Providencia stuartii	29914	3.0	1.7	2.8	2.0

SUBSTITUTE SHEET

123

	Pseudomonas aeruginosa	10145	2.0	1.9	1.3	2.0
	Pseudomonas fluorescens	13525	2.5	2.7	2.1	2.0
	Serratia marcescens	13880	2.5	1.7	1.8	2.0
	Staphylococcus aureus	12600	2.0	1.7	1.8	2.0
5	Staphylococcus					
	epidermidis	14990	3.0	1.5	1.3	2.0
	Streptococcus agalactiae	13813	2.5	1.9	1.3	2.5
	Streptococcus faecalis	19433	1.7	3.3	3.5	1.9
	Streptococcus faecium	19434	2.0	2.9	2.1	1.5
10	Ureaplasma urealyticum	27618	2.1	3.1	2.4	1.8

15

TABLE 71

HYBRIDIZATION OF FUNGAL PROBES 1-4 TO RNAs OF A CROSS
SECTION OF PHYLOGENETICALLY DIVERSE ORGANISMS

20	Organism	ATCC#	% Probe Bound			
			#1	#2	#3	#4
	Acinetobacter					
	calcoaceticus	23055	2.5	2.5	2.0	1.9
	Bacillus subtilis	6051	2.0	2.8	2.4	2.4
25	Bacteroides fragilis	23745	2.0	2.2	2.5	2.3
	Branhamella catarrhalis	25238	2.5	3.2	1.8	1.7
	Campylobacter jejuni	33560	2.5	2.1	2.0	1.9
	Chlamydia trachomatis	VR878	3.1	3.1	1.8	2.7
	Chromobacterium					
30	violaceum	29094	2.5	1.7	2.0	2.2
	Clostridium perfringens	13124	1.9	2.3	1.8	1.8
	Corynebacterium xerosis	373	1.6	4.8	1.8	1.1
	Deinococcus radiodurans	35073	2.0	1.6	2.1	0.8
	Derxia gummosa	15994	3.0	1.5	1.7	1.8
35	Gardnerella vaginalis	14018	2.0	2.2	1.3	1.2
	Hafnia alvei	13337	1.0	2.5	1.7	1.6
	Lactobacillus					
	acidophilus	4356	2.0	2.7	2.0	1.9
	Moraxella osloensis	19976	2.0	2.1	1.9	1.8
40	Mycobacterium smegmatis	14468	1.6	1.8	1.8	1.7
	Mycoplasma hominis	14027	1.5	1.8	1.6	1.5
	Neisseria gonorrhoeae	19424	2.0	2.7	1.6	1.6
	Rahnella aquatilis	33071	2.0	2.7	2.3	2.1
	Rhodospirillum rubrum	11170	2.0	1.8	1.6	1.5
45	Vibrio parahaemolyticus	17802	2.5	3.1	1.7	1.6
	Yersinia enterocolitica	9610	2.0	1.8	2.3	2.2
	Human		2.0	1.8	2.1	3.0

SUBSTITUTE SHEET

CGK00033258

Two derivatives of probe 1 also were made:

5 CCCGACCGTCCCTATTAATCATTACGATGGTCCTAGAAAC
 CCCGACCGTCCCTATTAATCATTACGATGG

The first derivative works well at 65°C, the second at 60°C.

Example 21

10 Gonorrhea is one of the most commonly reported
bacterial infections in the United States, with over two
million cases reported annually. This sexually
transmitted disease usually results in anterior
urethritis in males and involves the cervix in females.
15 While severe complications and even sterility can occur
in untreated individuals, asymptomatic infections are
common, resulting in carriers who unknowingly spread the
disease.

 The causative agent, Neisseria gonorrhoeae, is a
20 gram negative, oxidase positive diplococcus with
stringent growth requirements. The method used for
diagnosis depends on the site of infection and the
patient symptoms. Gonococcal urethritis in males is
diagnosed with good sensitivity and specificity using
25 gram stain. Culture, requiring 24-72 hours, usually
must be performed to confirm diagnosis of gonorrhea from
all females and asymptomatic males. Following the
detection of the organism from growth in culture,
Neisseria gonorrhoeae must be identified by further
30 tests such as carbohydrate degradation, coagglutination,
fluorescent antibody screens or chromogenic enzyme
substrate assays.

Neisseria gonorrhoeae is particularly difficult to
detect and distinguish using a nucleic acid probe
35 because it is very closely related to N. meningitidis.

Data published in Kingsbury, D.T., J. Bacteriol. 94:870-874 (1967) shows a DNA:DNA homology for the two species of approximately 80-94%. Under guidelines established by the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics, Int'l J. System. Bacteriol. 37:463-464 (1987), the phylogenetic definition of a species generally means 70% or greater DNA:DNA homology. Despite the fact that these organisms may be considered to be the same species under established principles, we were able to make probes capable of distinguishing them.

As expected, the rRNA homology between N. gonorrhoeae and N. meningitidis is even greater because of known conserved regions. We noted a 1.0% difference between the 16S and a 1.1% difference between the 23S rRNA sequences of N. gonorrhoeae and N. meningitidis using our sequencing data.

Making a probe for N. gonorrhoeae was complicated by the fact that in some sites where N. meningitidis and N. gonorrhoeae differed, other Neisseria species were similar to N. gonorrhoeae. The few mismatches which exist between these two species are in the most variable regions, i.e., regions which vary not only between species, but also from strain to strain. Despite the fact that some believed the species could not be distinguished with nucleic acid probes at all, and others believed that rRNA was too conserved to be useful in probe diagnostics, we were able to make probes capable of differentiating N. gonorrhoeae and N. meningitidis.

The present invention has significant advantages over each of the prior art methods; the probes are more specific and much faster than culture methods. It also is believed that the probes are more sensitive, (i.e., able to detect a smaller number of organisms in a clinical sample) than prior art methods.

The primers used to identify these probe sequences had the following sequences:

1. GGCCGTTACCCACCTACTAGCTAAT
2. GTATTACCGCGGCTGCTGGCAC
- 5 3. GCTCGTTGCGGGACTTAACCCACCAT

Each of the rRNA sites chosen to target had at least two mismatches to E. coli, N. meningitidis, N. cinerea, N. lactamica, N. mucosa, and Kingella kingae.

10 Oligonucleotides complementary to sequences adjacent to the probe regions were synthesized and used in the hybridization mix according to Hogan et al., U.S. Patent App. Ser. No. _____ (not yet assigned), to be filed November 24, 1987, entitled "Means and Method for Enhancing Nucleic Acid Hybridization (the "helper"

15 patent application).

The following sequences were characterized and shown to be specific for Neisseria gonorrhoeae. The phylogenetically nearest neighbors Neisseria meningitidis, N. lactamica, N. cinerea, N. mucosa, and

20 Kingella kingae were used for comparison with the N. gonorrhoeae sequence.

1. CCG CCG CTA CCC GGT AC
2. TCA TCG GCC GCC GAT ATT GGC
3. GAG CAT TCC GCA CAT GTC AAA ACC AGG TA

25 Sequence 1, complementary to 16S rRNA in the region 125-150, is 17 bases in length and has a T_m of 56°C. Sequence 2, complementary to 16S rRNA in the region 455-485, is 21 bases in length and has a T_m of 63°C. Sequence 3, complementary to 16S rRNA in the region 980-

30 1015, is 29 bases in length and has a T_m of 57°C.

The reactivity and specificity of the probes for Neisseria gonorrhoeae was demonstrated with a hybridization assay. The three oligonucleotide probes were iodinated and mixed with unlabeled oligonucleotides

35 of sequence 5'-CCC CTG CTT TCC CTC TCT AGA CGT ATG CGG

TAT TAG CTG ATC TTT CG-3', 5'-GCC TTT TCT TCC CTG ACA
 AAA GTC CTT TAC AAC CCG-3', 5'-GGC ACG TAG TTA GCC GGT
 GCT TAT TCT TCA GGT AC-3', and 5'-GGT TCT TCG CGT TGC
 ATC GAA TTA ATC CAC ATC ATC CAC CGC-3', and with
 5 purified RNA in 0.48 M sodium phosphate, pH6.8, 0.5%
 sodium dodecyl sulfate (SDS) and incubated at 60°C for
 one hour. Following incubation, 4 ml of 2%
 hydroxyapatite, 0.12 M sodium phosphate pH6.8, 0.02% SDS
 was added and the mixture was incubated at 60°C for 5
 10 minutes. The samples were centrifuged and the
 supernatants were removed. Five ml of wash solution
 (0.12 M sodium phosphate pH6.8, 2% SDS) was added and
 the samples were mixed, centrifuged, and the
 supernatants removed. The amount of radioactivity bound
 15 to the hydroxyapatite was determined in a gamma counter.

Table 72 shows that the probes hybridize well to N. gonorrhoeae RNA and do not hybridize to the other species tested.

20

TABLE 72

HYBRIDIZATION OF NEISSERIA GONORRHOEAE
 PROBES 1-3 TO NEISSERIA AND KINGELLA RNAS

25	<u>Organisms</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Kingella kingae	23332	0.09
	Neisseria cinerea	14685	0.04
	N. gonorrhoeae	19424	48.4
	N. lactamica	23970	0.07
30	N. meningitidis		
	serogroup A	13077	0.04
	N. meningitidis		
	serogroup B	13090	0.04
	N. meningitidis		
35	serogroup C	13102	0.04
	N. mucosa	19696	0.07
	N. subflava	14799	0.05

SUBSTITUTE SHEET

The following derivatives of Neisseria probes also have been made and used:

5 GAG GAT TCC GCA CAT GTC AAA ACC AGG
 GAG GAT TCC GCA CAT GTC AAA ACC AGG TAA
 CCC GCT ACC CGG TAC GTT C
 CCG CTA CCC GGT ACG TTC.

10

 Although the above examples of performance were determined using the standard assay format previously described, the specific probes may be used under a wide variety of experimental conditions. For example, additives may be included to the reaction solutions to provide optimal reaction conditions for accelerated hybridization. Such additives may include buffers, chelators, organic compounds and nucleic acid precipitating agents such as detergents, dihydroxybenzene, sodium dodecyl sulfate, sodium diisobutyl sulfosuccinate, sodium tetradecyl sulfate, sarkosyl and the alkali metal salts and ammonium salts of SO-2₄, PO-3₄, Cl-1 and HCOO-1. Such additives can be utilized by one skilled in the art to provide optimal conditions for the hybridization reaction to take place. These conditions for accelerated hybridization of single stranded nucleic acid molecules into double stranded molecules are the subject of the above-noted U.S. Patent App. Ser. No. 627,795 filed July 5, 1984, continuation filed June 4, 1987 (serial no. not yet assigned) and Ser. No. 816,711 filed January 7, 1986, which are both entitled ACCELERATED NUCLEIC ACID REASSOCIATION METHOD.

 The present invention can be carried out on nonviral organisms from purified samples or unpurified clinical samples such as sputum, feces, tissue, blood,

SUBSTITUTE SHEET

CGK00033263

spinal or synovial fluids serum, urine or other bodily fluids, or other samples such as environmental or food samples. Prior to cell breakage and hybridization, the cells can be suspended or placed in solution. In the case of the unpurified samples referred to above, the cells may remain intact and untreated in their own biological environment prior to the assay.

The probes of the present invention may be used in an assay either alone or in combination with different probes. Several individual probes also can be linked together during nucleic acid synthesis. This results in one probe molecule which contains multiple probe sequences, and therefore, multiple specificities. For example, a single nucleic acid molecule can be synthesized which contains both the Mycobacterium avium and the Mycobacterium intracellulare sequences described in Examples 1 and 2. When hybridized with either M. avium or M. intracellulare rRNA this probe will hybridize completely. If the two probe sequences were combined separately in an assay only one half of the mixed individual probes will hybridize with either M. avium or M. intracellulare rRNA. Other embodiments also may be practiced within the scope of the claims. For example, probes may be labelled using a variety of labels, as described within, and may be incorporated into diagnostic kits.

SUBSTITUTE SHEET

CGK00033264

We claim:

1. A method for preparing a probe for use in a qualitative or quantitative hybridization assay which comprises constructing an oligonucleotide that is sufficiently complementary to hybridize to a region of rRNA selected to be unique to a non-viral organism or group of non-viral organisms sought to be detected, said region of rRNA being selected by comparing one or more variable region rRNA sequences of said non-viral organism or group of non-viral organisms with one or more variable region rRNA sequences from one or more non-viral organisms sought to be distinguished therefrom.

2. The method of claim 1 wherein said variable region rRNA sequences from non-viral organisms sought to be distinguished are from the known nearest related organism to said non-viral organism or group of non-viral organisms sought to be detected.

3. The method of claim 1 wherein said region of rRNA is selected to have at least about a one base sequence difference from a corresponding rRNA sequence of the known nearest related organism to said non-viral organism or group of non-viral organisms sought to be detected.

4. The method of claim 1 wherein said region of rRNA is selected to have at least about a 10% or greater base sequence difference from the corresponding rRNA sequence of the known nearest related organism to said non-viral organism or group of non-viral organisms sought to be detected.

5. The method of claim 1 wherein said region of rRNA is chosen from the group consisting of 5S, 16S, and 23S rRNA.

6. The method of claim 1 wherein said

region of rRNA is chosen from the group consisting of 5.0S, 5.8S, 18S and 28S rRNA.

7. The method of claim 1 wherein said oligonucleotide is at least about 10 nucleotides in length.

8. The method of claim 1 wherein said oligonucleotide is at least about 15 nucleotides in length.

9. The method of claim 1 wherein said oligonucleotide is at least about 20 nucleotides in length.

10. The method of claim 1 wherein said oligonucleotide is at least about 30 nucleotides in length.

11. The method of claim 1 wherein said oligonucleotide is about 20 nucleotides to about 50 nucleotides in length.

12. The method of claim 1 wherein said oligonucleotide is about 30 nucleotides to about 50 nucleotides in length.

13. The method of claim 3 wherein said oligonucleotide is at least about 10 nucleotides in length.

14. The method of claim 3 wherein said oligonucleotide is at least about 15 nucleotides in length.

15. The method of claim 1 wherein said oligonucleotide is at least about 20 nucleotides in length.

16. The method of claim 3 wherein said oligonucleotide is at least about 30 nucleotides in length.

17. The method of claim 3 wherein said oligonucleotide is about 20 nucleotides to about 50 nucleotides in length.

18. The method of claim 3 wherein said oligonucleotide is at about 30 nucleotides to about 50 nucleotides in length.

5 19. The method of claim 4 wherein said oligonucleotide is at least about 10 nucleotides in length.

20. The method of claim 4 wherein said oligonucleotide is at least about 15 nucleotides in length.

10 21. The method of claim 4 wherein said oligonucleotide is at least about 20 nucleotides in length.

22. The method of claim 4 wherein said oligonucleotide is at least about 30 nucleotides in
15 length.

23. The method of claim 4 wherein said oligonucleotide is about 20 nucleotides to about 50 nucleotides in length.

24. The method of claim 4 wherein said
20 oligonucleotide is about 30 nucleotides to about 50 nucleotides in length.

25. The method of claim 1 wherein said probe is at least about 75% complementary to said region of rRNA.

25 26. The method of claim 3 wherein said oligonucleotide is at least about 75% complementary to said region of rRNA.

27. The method of claim 4 wherein said oligonucleotide is at least about 75% complementary to said region of rRNA.

28. The method of claim 1 wherein said probe
5 is perfectly complementary to said region of rRNA.

29. The method of claim 3 wherein said probe is perfectly complementary to said region of rRNA.

30. The method of claim 4 wherein said probe is perfectly complementary to said region of rRNA.

10 31. A hybridization assay probe for a non-viral organism or organisms comprising an oligonucleotide of at least about 10 nucleotides in length wherein at least about 10 contiguous nucleotides are substantially complementary to at least one variable
15 region of nucleic acid selected to be unique to said non-viral organism or organisms.

32. A hybridization assay probe for a non-viral organism or organisms comprising an oligonucleotide of at least about 10 nucleotides in
20 length which is at least about 75% complementary to at least one variable region of nucleic acid selected to be unique to said non-viral organism or organisms.

33. The probe of claim 31 or 32 wherein said nucleic acid is 5S, 16S, or 23S rRNA.

25 34. The probe of claim 31 or 32 wherein said nucleic acid is 5.0S, 5.8S, 18S, or 28S rRNA.

35. The probe of claim 31 wherein said non-viral organism is Mycobacterium avium.

36. The probe of claim 35 wherein said oligonucleotide comprises the sequence
5 ACCGCAAAAGCTTTCCACCAGAAGACATGCGTCTTGAG.

37. A nucleotide polymer capable of hybridizing to the probe of claim 36 or to the complement thereof.

38. A nucleic acid hybrid formed between an
10 oligonucleotide comprising the sequence

ACCGCAAAAGCTTTCCACCAGAAGACATGCGTCTTGAG and a nucleic acid sequence substantially complementary thereto.

39. A nucleotide polymer of the structure
15 ACCGCAAAAGCTTTCCACCAGAAGACATGCGTCTTGAG and the complement thereto.

40. A nucleotide polymer capable of hybridizing to RNA of the species Mycobacterium avium in the region corresponding to bases 185-225 of E. coli 16S
20 rRNA.

41. A nucleic acid hybrid formed between a nucleotide polymer of claim 40 and a nucleic acid sequence substantially complementary thereto.

42. The probe of claim 31 wherein said non-viral organism is Mycobacterium intracellulare.
25

SECRET

135

43. The probe of claim 42 wherein said oligonucleotide comprises the sequence

ACCGCAAAAGCTTTCCACCTAAAGACATGCGCCTAAAG.

44. A nucleotide polymer capable of hybridizing to the probe of claim 43 or to the complement thereof.

45. A nucleic acid hybrid formed between an oligonucleotide comprising the sequence

ACCGCAAAAGCTTTCCACCTAAAGACATGCGCCTAAAG

and a nucleic acid sequence substantially complementary thereto.

46. A nucleotide polymer of the structure ACCGCAAAAGCTTTCCACCTAAAGACATGCGCCTAAAG and the complement thereto.

47. A nucleotide polymer capable of hybridizing to the RNA of the species Mycobacterium intracellulare in the region corresponding to bases 185-225 of E. coli 16S rRNA.

48. A nucleic acid hybrid formed between a nucleotide polymer of claim 47 and a nucleic acid sequence substantially complementary thereto.

49. The probe of claim 31 wherein said non-viral organisms are the Mycobacterium tuberculosis-complex bacteria.

50. The probe of claim 49 wherein said

SUBSTITUTE SHEET

CGK00033270

oligonucleotide comprises the sequence
TAAAGCGCTTTCCACCACAAGACATGCATCCCGTG.

51. The probe of claim 49 wherein said
oligonucleotide comprises the sequence
5 TGCCCTACCCACACCCACCACAAGGTGATGT.

52. The probe of claim 49 wherein said
oligonucleotide comprises the sequence
CCATCACCACCTCCTCCGGAGAGGAAAAGG.

53. The probe of claim 49 wherein said
10 oligonucleotide comprises the sequence
CTGTCCCTAAACCCGATTGAGGGTTCGAGGTTAGATGC.

54. The probe of claim 49 wherein said
oligonucleotide comprise the sequence
AGGCACTGTCCCTAAACCCGATT CAGGGTTC.

15 55. The probe of claim 49 wherein said
oligonucleotide comprises the sequence
CCGCTAAAGCGCTTTCCACCACAAGACATGCATCCCG.

56. The probe of claim 49 wherein said
oligonucleotide comprise the sequence
20 ACACCGCTAAAGCGCTTTCCACCACAAGACATGCATC.

57. A nucleotide polymer capable of
hybridizing to the probe of claims 50 or 51 or 52 or 53
or 54 or 55 or 56 or to the complements thereof.

58. A nucleic acid hybrid formed between an
25 oligonucleotide comprising a member of the group
consisting of oligonucleotides of the sequences

TAAAGCGCTTTCCACCACAAGACATGCATCCCGTG,
TGCCCTACCCACACCCACCACAAGGTGATGT,
CCATCACCACCTCCTCCGGAGAGGAAAAGG,
CTGTCCCTAAACCCGATTTCAGGGTTCGAGGTTAGATGC,
5 AGGCACTGTCCCTAAACCCGATTTCAGGGTTC,
CCGCTAAAGCGCTTTCCACCACAAGACATGCATCCCG, and
ACACCGCTAAAGCGCTTTCCACCACAAGACATGCATC;

and a nucleic acid sequence substantially complementary thereto.

- 10 59. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the structures

TAAAGCGCTTTCCACCACAAGACATGCATCCCGTG,
TGCCCTACCCACACCCACCACAAGGTGATGT,
15 CCATCACCACCTCCTCCGGAGAGGAAAAGG,
CTGTCCCTAAACCCGATTTCAGGGTTCGAGGTTAGATGC,
AGGCACTGTCCCTAAACCCGATTTCAGGGTTC,
CCGCTAAAGCGCTTTCCACCACAAGACATGCATCCCG, and
ACACCGCTAAAGCGCTTTCCACCACAAGACATGCATC;

- 20 and the complements thereto.

60. A nucleotide polymer capable of hybridizing to the rRNA of the species included in the Mycobacterium tuberculosis complex in the region corresponding to bases 185-225 of E. coli 16S rRNA.

- 25 61. A nucleic acid hybrid formed between a

nucleotide polymer of claim 60 and a nucleic acid substantially complementary thereto.

62. A nucleotide polymer capable of hybridizing to the RNA of the species included in the
5 Mycobacterium tuberculosis complex in the region corresponding to bases 540-575 of E. coli 23S rRNA.

63. A nucleic acid hybrid formed between a nucleotide polymer of claim 62 and a nucleic acid substantially complementary thereto.

10 64. A nucleotide polymer capable of hybridizing to the RNA of the species included in the Mycobacterium tuberculosis complex in the region corresponding to bases 1155-1190 of E. coli 23S rRNA.

65. A nucleic acid hybrid formed between a
15 nucleotide polymer of claim 64 and a nucleic acid substantially complementary thereto.

66. A nucleotide polymer capable of hybridizing to the RNA of the species included in the Mycobacterium tuberculosis complex in the region
20 corresponding to bases 2195-2235 of E. coli 23S rRNA.

67. A nucleic acid hybrid formed between a nucleotide polymer of claim 66 and a nucleic acid substantially complementary thereto.

68. The probe of claim 31 wherein said non-
25 viral organisms are the genus Mycobacterium.

69. The probe of claim 68 wherein said oligonucleotide comprises the sequence CCA TGC ACC ACC TGC ACA CAG GCC ACA AGG.

70. The probe of claim 68 wherein said oligonucleotide comprises the sequence GGC TTG CCC CAG TAT TAC CAC TGA CTG GTA CGG.

71. The probe of claim 68 wherein said oligonucleotide comprises the sequence CAC CGA ATT CGC CTC AAC CGG CTA TGC GTC ACC TC.

72. The probe of claim 68 wherein said oligonucleotide comprises the sequence GGG GTA CGG CCC GTG TGT GTG CTC GCT AGA GGC.

73. A nucleotide polymer capable of hybridizing to the probes of claims 69 or 70 or 71 or 72 or to the complements thereof.

74. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequence

CCA TGC ACC ACC TGC ACA CAG GCC ACA AGG,
GGC TTG CCC CAG TAT TAC CAC TGA CTG GTA CGG,
CAC CGA ATT CGC CTC AAC CGG CTA TGC GTC ACC
TC, and

GGG GTA CGG CCC GTG TGT GTG CTC GCT AGA GGC;
and a nucleic acid sequence substantially complementary thereto.

75. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the structures

CCA TGC ACC ACC TGC ACA CAG GCC ACA AGG,
5 GGC TTG CCC CAG TAT TAC CAC TGA CTG GTA CGG,
CAC CGA ATT CGC CTC AAC CGG CTA TGC GTC ACC
TC, and

GGG GTA CGG CCC GTG TGT GTG CTC GCT AGA GGC;
and the complements thereto.

10 76. A nucleotide polymer capable of hybridizing to RNA of the genus Mycobacterium in the region corresponding to bases 1025-1060 of E. coli 16S rRNA.

15 77. A nucleic acid hybrid formed between a nucleotide polymer of claim 76 and a nucleic acid sequence substantially complementary thereto.

78. A nucleotide polymer capable of hybridizing to RNA of the genus Mycobacterium in the region corresponding to bases 1440-1475 of E. coli 23S
20 rRNA.

79. A nucleic acid hybrid formed between a nucleotide polymer of claim 78 and a nucleic acid sequence substantially complementary thereto.

80. A nucleotide polymer capable of
25 hybridizing to RNA of the genus Mycobacterium in the

region corresponding to bases 1515-1555 of E. coli 23S rRNA.

81. A nucleic acid hybrid formed between a nucleotide polymer of claim 80 and a nucleic acid sequence substantially complementary thereto.

82. A nucleotide polymer capable of hybridizing to RNA of the genus Mycobacterium in the region corresponding to bases 1570-1610 of E. coli 23S rRNA.

83. A nucleic acid hybrid formed between a nucleotide polymer of claim 82 and a nucleic acid sequence substantially complementary thereto.

84. The probe of claim 31 wherein said non-viral organism is Mycoplasma pneumoniae.

85. The probe of claim 84 wherein said oligonucleotide comprises the sequence
GCTTGGTGCTTTCCTATTCTCACTGAAACAGCTACATTCGGC.

86. The probe of claim 84 wherein said oligonucleotide comprises the sequence
AATAACGAACCCTTGCAGGTCCTTTCAACTTTGAT.

87. The probe of claim 84 wherein said oligonucleotide comprises the sequence
CAGTCAAACCTAGCCATTACCT GCTAAAGTCATT.

88. The probe of claim 84 wherein said oligonucleotide comprise the sequence
TACCGAGGGGATCGCCCCGACAGCTAGTAT.

89. The probe of claim 84 wherein said oligonucleotide comprises the sequence CTTTACAGATTTGCTCACTTTTACAAGCTGGCGAC.

90. A nucleotide polymer capable of hybridizing to the probes of claims 85 or 86 or 87 or 88 or 89 or to the complements thereof.

91. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequence

10 GCTTGGTGCTTTCCTATTCTCACTGAAACAGCTACATTCCGGC,
AATAACGAACCCTTGCAGGTCCTTTCAACTTTGAT,
CAGTCAAACCTCTAGCCATTACCTGCTAAAGTCATT,
TACCGAGGGGATCGCCCCGACAGCTAGTAT, and
CTTTACAGATTTGCTCACTTTTACAAGCTGGCGAC;

15 and a nucleic acid sequence substantially complementary thereto.

92. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the structures

20 GCTTGGTGCTTTCCTATTCTCACTGAAACAGCTACATTCCGGC,
AATAACGAACCCTTGCAGGTCCTTTCAACTTTGAT,
CAGTCAAACCTCTAGCCATTACCTGCTAAAGTCATT,
TACCGAGGGGATCGCCCCGACAGCTAGTAT, and
CTTTACAGATTTGCTCACTTTTACAAGCTGGCGAC;

25 and the complements thereto.

93. A nucleotide polymer capable of hybridizing to the RNA of the species Mycoplasma pneumoniae in the region corresponding to bases 190-230 of E. coli 16S rRNA.

5 94. A nucleic acid hybrid formed between a nucleotide polymer of claim 93 and a nucleic acid sequence substantially complementary thereto.

95. A nucleotide polymer capable of hybridizing to the RNA of the species Mycoplasma pneumoniae in the region corresponding to bases 450-490 of E. coli 16S rRNA.

96. A nucleic acid hybrid formed between a nucleotide polymer of claim 95 and a nucleic acid sequence substantially complementary thereto.

15 97. A nucleotide polymer capable of hybridizing to the RNA of the species Mycoplasma pneumoniae in the region corresponding to bases 820-860 of E. coli 16S rRNA.

98. A nucleic acid hybrid formed between a
20 nucleotide polymer of claim 97 and a nucleic acid sequence substantially complementary thereto.

99. A nucleotide polymer capable of hybridizing to the RNA of the species Mycoplasma pneumoniae in the region corresponding to bases 1255-
25 1290 of E. coli 16S rRNA.

100. A nucleic acid hybrid formed between a nucleotide polymer of claim 99 and a nucleic acid sequence substantially complementary thereto.

101. A nucleotide polymer capable of
5 hybridizing to the RNA of the species Mycoplasma pneumoniae in the region corresponding to bases 65-120 of E. coli 5S rRNA.

102. A nucleic acid hybrid formed between a nucleotide polymer of claim 101 and a nucleic acid
10 sequence substantially complementary thereto.

103. The probe of claim 31 wherein said non-viral organisms are the genus Legionella.

104. The probe of claim 103 wherein said oligonucleotide comprises the sequence
15 TACCCTCTCCCACTCGAGT CAACCAGTATTATCTGACC.

105. The probe of claim 103 wherein said oligonucleotide comprises the sequence GGATTTACAGTGTCCCGGCCTACTT GTTCGGGTGCGTAGTTC.

106. The probe of claim 103 wherein said
20 oligonucleotide comprises the sequence CATCTCTGCAAATTCAGTGTAT GTCAAGGGTAGGTAAGG.

107. The probe of claim 103 wherein said oligonucleotide comprises the sequence GCGGTACGGTTCTCTATAA GTTATGGCTAGC.

25 108. The probe of claim 103 wherein said

145

oligonucleotide comprises the sequence
GTACCGAGGGTACCTTTGTGCT.

109. The probe of claim 103 wherein said
oligonucleotide comprises the sequence
5 CACTCTTGGTACGATGTCCGAC.

110. A nucleotide polymer capable of
hybridizing to the probes of claims 104 or 105 or 106 or
107 or 108 or 109 or to the complements thereof.

111. A nucleic acid hybrid formed between an
10 oligonucleotide comprising a member of the group
consisting of oligonucleotides of the sequences

TACCCTCTCCCATACTCGAGTCAACCAGTATTATCTGACC,
GGATTTACGTGTCCCGGCCTACTTGTTTCGGGTGCGTAGTTC,
CATCTCTGCAAAATTCAGTGTATGTCAAGGGTAGGTAAGG,
15 GCGGTACGGTTCTCTATAAGTTATGGCTAGC,
GTACCGAGGGTACCTTTGTGCT, and
CACTCTTGGTACGATGTCCGAC;

and a nucleic acid sequence substantially complementary
thereto.

20 112. A nucleotide polymer comprising a
member of the group consisting of nucleotide polymers of
the structures

TACCCTCTCCCATACTCGAGTCAACCAGTATTATCTGACC,
GGATTTACGTGTCCCGGCCTACTTGTTTCGGGTGCGTAGTTC,
CATCTCTGCAAAATTCAGTGTATGTCAAGGGTAGGTAAGG,
25 GCGGTACGGTTCTCTATAAGTTATGGCTAGC,

GTACCGAGGGTACCTTTGTGCT, and

CACTCTTGGTACGATGTCCGAC;

and the complements thereto.

113. A nucleotide polymer capable of
5 hybridizing to the RNA of the genus Legionella in the
region corresponding to bases 630-675 of E. coli 16S
rRNA.

114. A nucleic acid hybrid formed between a
nucleotide polymer of claim 113 and a nucleic acid
10 sequence substantially complementary thereto.

115. A nucleotide polymer capable of
hybridizing to the RNA of the genus Legionella in the
region corresponding to bases 975-1020 of E. coli 16S
rRNA.

15 116. A nucleic acid hybrid formed between a
nucleotide polymer of claim 115 and a nucleic acid
sequence substantially complementary thereto.

117. A nucleotide polymer capable of
hybridizing to the RNA of the genus Legionella in the
20 region corresponding to bases 350-395 of E. coli 23S
rRNA.

118. A nucleic acid hybrid formed between a
nucleotide polymer of claim 117 and a nucleic acid
sequence substantially complementary thereto.

25 119. A nucleotide polymer capable of
hybridizing to the RNA of the genus Legionella in the

region corresponding to bases 1585-1620 of E. coli 23S rRNA.

120. A nucleic acid hybrid formed between a nucleotide polymer of claim 119 and a nucleic acid sequence substantially complementary thereto.

121. A nucleotide polymer capable of hybridizing to the RNA of the genus Legionella in the region corresponding to bases 2280-2330 of E. coli 23S rRNA.

122. A nucleic acid hybrid formed between a nucleotide polymer of claim 121 and a nucleic acid sequence substantially complementary thereto.

123. The probe of claim 31 wherein said non-viral organism is Chlamydia trachomatis.

124. The probe of claim 123 wherein said oligonucleotide comprises the sequence
CCGACTCGGGGTTGAGCCCATCTTTGACAA.

125. The probe of claim 123 wherein said oligonucleotide comprise the sequence
TTACGTCCGACACGGATGGGGTTGAGACCATC.

126. The probe of claim 123 wherein said oligonucleotide comprises the sequence
CCGCCACTAAACAATCGTCGAAACAATTGCTCCGTTCGA.

127. The probe of claim 123 wherein said oligonucleotide comprises the sequence
CGTTACTCGGATGCCCAAATATCGCCACATTCG.

128. The probe of claim 123 wherein said oligonucleotide comprises the sequence
CATCCATCTTTCCAGATGTGTTCAACTAGGAGTCCTGATCC.

5 129. The probe of claim 123 wherein said oligonucleotide comprises the sequence
GAGGTCGGTCTTTCTCTCCTTTCTGCTACG.

130. The probe of claim 123 wherein said oligonucleotide comprises the sequence
CCGTTCTCATCGCTCTACGGACTCTTCCAATCG.

10 131. The probe of claim 123 wherein said oligonucleotide comprises the sequence
CGAAGATTCCCCTTGATCGCGACCTGATCT.

132. The probe of claim 123 wherein said oligonucleotide comprises the sequence
15 CCGGGGCTCCTATCGTTCCATAGTCACCCTAAAAG.

133. The probe of claim 123 wherein said oligonucleotide comprises the sequence
TACCGCGTGTCTTATCGACACACCCGCG.

134. A nucleotide polymer capable of
20 hybridizing to the probes of claims 124 or 125 or 126 or 127 or 128 or 129 or 130 or 131 or 132 or 133 or to the complements thereof.

135. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group
25 consisting of oligonucleotides of the sequence

CCGACTCGGGGTTGAGCCCATCTTTGACAA,

TTACGTCCGACACGGATGGGGTTGAGACCATC,
CCGCCACTAAACAATCGTCGAAACAATTGCTCCGTTCTGA,
CGTTACTCGGATGCCCAAATATCGCCACATTCTG,
CATCCATCTTTCCAGATGTGTTCAACTAGGAGTCCTGATCC,
5 GAGGTCGGTCTTTCTCTCCTTTTCGTCTACG,
CCGTTCTCATCGCTCTACGGACTCTTCCAATCG,
CGAAGATTCCCCTTGATCGCGACCTGATCT,
CCGGGGCTCCTATCGTTCCATAGTCACCCTAAAAG, and
TACCGCGTGTCTTATCGACACACCCGCG;

10 and a nucleic acid sequence substantially complementary thereto.

136. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the structures

15 CCGACTCGGGGTTGAGCCCATCTTTGACAA,
TTACGTCCGACACGGATGGGGTTGAGACCATC,
CCGCCACTAAACAATCGTCGAAACAATTGCTCCGTTCTGA,
CGTTACTCGGATGCCCAAATATCGCCACATTCTG,
CATCCATCTTTCCAGATGTGTTCAACTAGGAGTCCTGATCC,
20 GAGGTCGGTCTTTCTCTCCTTTTCGTCTACG,
CCGTTCTCATCGCTCTACGGACTCTTCCAATCG,
CGAAGATTCCCCTTGATCGCGACCTGATCT,
CCGGGGCTCCTATCGTTCCATAGTCACCCTAAAAG, and
TACCGCGTGTCTTATCGACACACCCGCG;

25 and the complements thereto.

137. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 60-105 of E. coli 16S rRNA.

5 138. A nucleic acid hybrid formed between a nucleotide polymer of claim 137 and a nucleic acid substantially complementary thereto.

139. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 175-210 of E. coli 16S rRNA.

140. A nucleic acid hybrid formed between a nucleotide polymer of claim 139 and a nucleic acid substantially complementary thereto.

15 141. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 600-635 of E. coli 16S rRNA.

142. A nucleic acid hybrid formed between a nucleotide polymer of claim 141 and a nucleic acid substantially complementary thereto.

143. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 830-870 of E. coli 16S rRNA.

144. A nucleic acid hybrid formed between a nucleotide polymer of claim 143 and a nucleic acid substantially complementary thereto.

145. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 275-320 of E. coli 23S rRNA.

146. A nucleic acid hybrid formed between a nucleotide polymer of claim 145 and a nucleic acid substantially complementary thereto.

147. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 330-365 of E. coli 23S rRNA.

148. A nucleic acid hybrid formed between a nucleotide polymer of claim 147 and a nucleic acid substantially complementary thereto.

149. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 1160-1190 of E. coli 23S rRNA.

150. A nucleic acid hybrid formed between a nucleotide polymer of claim 149 and a nucleic acid substantially complementary thereto.

151. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia

trachomatis in the region corresponding to bases 1450-1490 of E. coli 23S rRNA.

152. A nucleic acid hybrid formed between a nucleotide polymer of claim 151 and a nucleic acid substantially complemen-tary thereto.

153. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 1510-1545 of E. coli 23S rRNA.

154. A nucleic acid hybrid formed between a nucleotide polymer of claim 153 and a nucleic acid substantially complemen-tary thereto.

155. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 1710-1750 of E. coli 23S rRNA.

156. A nucleic acid hybrid formed between a nucleotide polymer of claim 155 and a nucleic acid substantially complementary thereto.

157. The probe of claim 31 wherein said non-viral organism is Campylobacter.

158. The probe of claim 157 wherein said oligonucleotide comprises the sequence

CGC TCC GAA AAG TGT CAT CCT CC.

159. The probe of claim 157 wherein said oligonucleotide comprises the sequence

CCT TAG GTA CCG TCA GAA TTC TTC CC.

160. The probe of claim 157 wherein said oligonucleotide comprises the sequence

GCC TTC GCA ATG GGT ATT CTT GGTG.

5 161. The probe of claim 157 wherein said oligonucleotide comprises the sequence

GGT TCT TAG GAT ATC AAG CCC AGG.

162. A nucleotide polymer capable of hybridizing to the probes of claims 158 or 159 or 160 or
10 161 or to the complements thereof.

163. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequence

15 CGC TCC GAA AAG TGT CAT CCT CC,
CCT TAG GTA CCG TCA GAA TTC TTC CC,
GCCTTCGCAATGGGTATTCTTGGTG, and
GGT TCT TAG GAT ATC AAG CCC AGG;

and a nucleic acid sequence substantially complementary thereto.

20 164. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the structures

CGC TCC GAA AAG TGT CAT CCT CC,
CCT TAG GTA CCG TCA GAA TTC TTC CC,
25 GCCTTCGCAATGGGTATTCTTGGTG, and
GGT TCT TAG GAT ATC AAG CCC AGG;

and the complements thereto.

165. A nucleotide polymer capable of hybridizing to the RNA of the genus Campylobacter in the region corresponding to bases 405-428 of E. coli 16S rRNA.

166. A nucleic acid hybrid formed between a nucleotide polymer of claim 165 and a nucleic acid substantially complementary thereto.

167. A nucleotide polymer capable of hybridizing to the RNA of the genus Campylobacter in the region corresponding to bases 440-475 of E. coli 16S rRNA.

168. A nucleic acid hybrid formed between a nucleotide polymer of claim 167 and a nucleic acid substantially complementary thereto.

169. A nucleotide polymer capable of hybridizing to the RNA of the genus Campylobacter in the region corresponding to bases 705-735 of E. coli 16S rRNA.

170. A nucleic acid hybrid formed between a nucleotide polymer of claim 169 and a nucleic acid substantially complementary thereto.

171. A nucleotide polymer capable of hybridizing to the RNA of the genus Campylobacter in the region corresponding to bases 980-1010 of E. coli 16S rRNA.

172. A nucleic acid hybrid formed between a nucleotide polymer of claim 171 and a nucleic acid sequence substantially complementary thereto.

173. The probe of claim 31 wherein said non-viral organisms are the sub-generic group of Streptococci known as enterococci.

174. The probe of claim 173 wherein said oligonucleotide comprises the sequence TGC AGC ACT GAA GGG CGG AAA CCC TCC AAC ACT TA.

10 175. A nucleotide polymer capable of hybridizing to the probe of claim 174 or to the complement thereof.

176. A nucleic acid hybrid formed between an oligonucleotide comprising the sequence

15 TGC AGC ACT GAA GGG CGG AAA CCC TCC AAC ACT TA and a nucleic acid sequence substantially complementary thereto.

177. A nucleotide polymer of the structure

20 TGC AGC ACT GAA GGG CGG AAA CCC TCC AAC ACT TA and the complement thereto.

178. A nucleotide polymer capable of hybridizing to the RNA of the sub-generic group Streptococci known as enterococci in the region corresponding to bases 825-860 of E. coli 16S rRNA.

25 179. A nucleic acid hybrid formed between a

nucleotide polymer of claim 178 and a nucleic acid sequence substantially complementary thereto.

180. The probe of claim 31 wherein said non-viral organisms are the subgeneric grouping known as
5 Group I *Pseudomonas*.

181. The probe of claim 180 wherein said oligonucleotide comprises the sequence

CAG ACA AAG TTT CTC GTG CTC CGT CCT ACT CGA
TT.

10 182. A nucleotide polymer capable of hybridizing to the probe of claim 181 or to the complement thereof.

183. A nucleic acid hybrid formed between an oligonucleotide comprising the sequence

15 CAG ACA AAG TTT CTC GTG CTC CGT CCT ACT CGA TT
and a nucleic acid substantially complementary thereto.

184. A nucleotide polymer of the structure

CAG ACA AAG TTT CTC GTG CTC CGT CCT ACT CGA TT
and the complement thereto.

20 185. A nucleotide polymer capable of hybridizing to the RNA of the sub-generic grouping known as group I *Pseudomonas* in the region corresponding to the bases 365-405 of *E. coli* 23S rRNA.

25 186. A nucleic acid hybrid formed between a nucleotide polymer of claim 185 and a nucleic acid sequence substantially complementary thereto.

187. The probe of claim 31 wherein said non-viral organism is Enterobacter cloacae.

188. The probe of claim 187 wherein said oligonucleotide comprises the sequence

5 GTG TGT TTT CGT GTA CGG GAC TTT CAC CC.

189. A nucleotide polymer capable of hybridizing to the probe of claim 189 or to the complement thereof.

10 190. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequence

GTG TGT TTT CGT GTA CGG GAC TTT CAC CC

and a nucleic acid sequence substantially complementary thereto.

15 191. A nucleotide polymer of the structure

GTG TGT TTT CGT GTA CGG GAC TTT CAC CC

and the complement thereto.

192. A nucleotide polymer capable of hybridizing to the RNA of the species Enterobacter cloacae in the region corresponding to bases 305-340 of E. coli 23S rRNA.

193. A nucleic acid hybrid formed between a nucleotide polymer of claim 192 and a nucleic acid sequence substantially complementary thereto.

25 194. The probe of claim 31 wherein said non-viral organism is Proteus mirabilis.

195. The probe of claim 194 wherein said oligonucleotide comprises the sequence

CCG TTC TCC TGA CAC TGC TAT TGA TTA AGA CTC.

196. A nucleotide polymer capable of
5 hybridizing to the probe of claim 195 or to the complement thereof.

197. A nucleic acid hybrid formed between an oligonucleotide comprising the sequence

CCG TTC TCC TGA CAC TGC TAT TGA TTA AGA CTC

10 and a nucleic acid sequence substantially complementary thereto.

198. A nucleotide polymer of the structure

CCG TTC TCC TGA CAC TGC TAT TGA TTA AGA CTC

and the complement thereto.

15 199. A nucleotide polymer capable of hybridizing to the RNA of the species Proteus mirabilis in the region corresponding to bases 270-305 of E. coli 23S rRNA.

200. A nucleic acid hybrid formed between a
20 nucleotide polymer of claim 199 and a nucleic acid sequence substantially complementary thereto.

201. The probe of claim 31 wherein said non-viral organisms are the genus Salmonella.

202. The probe of claim 201 wherein said
25 oligonucleotide comprises the sequence CTC CTT TGA GTT CCC GAC CTA ATC GCT GGC.

203. The probe of claim 201 wherein said oligonucleotide comprises the sequence CTC ATC GAG CTC ACA GCA CAT GCG CTT TTG TGT A.

204. A nucleotide polymer capable of hybridizing to the probe of claim 202 or 203 or to the complement thereof.

205. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequence

10 CTC CTT TGA GTT CCC GAC CTA ATC GCT GGC and
 CTC ATC GAG CTC ACA GCA CAT GCG CTT TTG TGT A;
and a nucleic acid sequence substantially complementary thereto.

206. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the structures

 CTC CTT TGA GTT CCC GAC CTA ATC GCT GGC and
 CTC ATC GAG CTC ACA GCA CAT GCG CTT TTG TGT A;
and the complements thereto.

20 207. A nucleotide polymer capable of hybridizing to the RNA of the genus Salmonella in the region corresponding to bases 1125-1155 of E. coli 16S rRNA.

25 208. A nucleic acid hybrid formed between a nucleotide polymer of claim 207 and a nucleic acid sequence substantially complementary thereto.

209. A nucleotide polymer capable of hybridizing to the RNA of the genus Salmonella in the region corresponding to bases 335-375 of E. coli 23S rRNA.

5 210. A nucleic acid hybrid formed between a nucleotide polymer of claim 209 and a nucleic acid sequence substantially complementary thereto.

211. The probe of claim 31 wherein said non-viral organism is Escherichia coli.

10 212. The probe of claim 211 wherein said oligonucleotide comprises the sequence

GCA CAT TCT CAT CTC TGA AAA CTT CCG TGG.

213. A nucleotide polymer capable of hybridizing to the probe of claim 212 or to the complement thereof.

214. A nucleic acid hybrid formed between an oligonucleotide comprising the sequence

GCA CAT TCT CAT CTC TGA AAA CTT CCG TGG

and a nucleic acid substantially complementary thereto.

20 215. A nucleotide polymer of the structure GCA CAT TCT CAT CTC TGA AAA CTT CCG TGG and the complement thereto.

216. A nucleotide polymer capable of hybridizing to the RNA of the species Escherichia coli in the region corresponding to bases 995-1030 of E. coli 16 sRNA.

217. A nucleic acid hybrid formed between a nucleotide polymer of claim 216 and a nucleic acid sequence substantially complementary thereto.

218. The probe of claim 31 wherein said non-viral organisms are the phylogenetic group bacteria.

219. The probe of claim 218 wherein said oligonucleotide comprises the sequence CCA CTG CTG CCT CCC GTA GGA GTC TGG GCC.

220. The probe of claim 218 wherein said oligonucleotide comprises the sequence CCA GAT CTC TAC GCA TTT CAC CGC TAC ACG TGG.

221. The probe of claim 218 wherein said oligonucleotide comprises the sequence GCT CGT TGC GGG ACT TAA CCC AAC AT.

222. The probe of claim 218 wherein said oligonucleotide comprises the sequence GGG GTT CTT TTC GCC TTT CCC TCA CGG.

223. The probe of claim 218 wherein said oligonucleotide comprises the sequence GGC TGC TTC TAA GCC AAC ATC CTG.

224. The probe of claim 218 wherein said oligonucleotide comprises the sequence GGA CCG TTA TAG TTA CGG CCG CC.

225. The probe of claim 218 wherein said oligonucleotide comprises the sequence GGT CGG AAC TTA CCC GAC AAG GAA TTT CGC TAC C.

226. A nucleotide polymer capable of hybridizing to the probes of claim 219 or 220 or 221 or 222 or 223 or 224 or 225 or to the complements thereof.

227. A nucleic acid hybrid formed between an
5 oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequences

CCA CTG CTG CCT CCC GTA GGA GTC TGG GCC,
CCA GAT CTC TAC GCA TTT CAC CGC TAC ACG TGG,
GCT CGT TGC GGG ACT TAA CCC AAC AT,
10 GGG GTT CTT TTC GCC TTT CCC TCA CGG,
GGC TGC TTC TAA GCC AAC ATC CTG,
GGA CCG TTA TAG TTA CGG CCG CC, and
GGT CGG AAC TTA CCC GAC AAG GAA TTT CGC TAC C;
and a nucleic acid sequence substantially similar
15 thereto.

228. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the structures

CCA CTG CTG CCT CCC GTA GGA GTC TGG GCC,
20 CCA GAT CTC TAC GCA TTT CAC CGC TAC ACG TGG,
GCT CGT TGC GGG ACT TAA CCC AAC AT,
GGG GTT CTT TTC GCC TTT CCC TCA CGG,
GGC TGC TTC TAA GCC AAC ATC CTG,
GGA CCG TTA TAG TTA CGG CCG CC, and
25 GGT CGG AAC TTA CCC GAC AAG GAA TTT CGC TAC C;
and the complements thereto.

229. A nucleotide polymer capable of hybridizing to the RNA of the phylogenetic group bacteria in the region corresponding to bases 330-365 of E. coli 16S rRNA.

5 230. A nucleic acid hybrid formed between a nucleotide polymer of claim 229 and a nucleic acid sequence substantially complementary thereto.

231. A nucleotide polymer capable of hybridizing to the RNA of the phylogenetic group bacteria in the region corresponding to bases 675-715 of E. coli 16S rRNA.

232. A nucleic acid hybrid formed between a nucleotide polymer of claim 231 and a nucleic acid sequence substantially complementary thereto.

15 233. A nucleotide polymer capable of hybridizing to the RNA of the phylogenetic group bacteria in the region corresponding to bases 1080-1110 of E. coli 16S rRNA.

234. A nucleic acid hybrid formed between a nucleotide polymer of claim 233 and a nucleic acid sequence substantially complementary thereto.

235. A nucleotide polymer capable of hybridizing to the RNA of the phylogenetic group bacteria in the region corresponding to bases 460-490 of E. coli 23S rRNA.

25

236. A nucleic acid hybrid formed between a nucleotide polymer of claim 235 and a nucleic acid sequence substantially complementary thereto.

237. A nucleotide polymer capable of
5 hybridizing to the RNA of the phylogenetic group bacteria in the region corresponding to bases 1050-1080 of E. coli 23S rRNA.

238. A nucleic acid hybrid formed between a nucleotide polymer of claim 237 and a nucleic acid
10 sequence substantially complementary thereto.

239. A nucleotide polymer capable of hybridizing to the RNA of the phylogenetic group bacteria in the region corresponding to bases 1900-1960 of E. coli 23S rRNA.

15 240. A nucleic acid hybrid formed between a nucleotide polymer of claim 239 and a nucleic acid sequence substantially complementary thereto.

241. The probe of claim 31 wherein said non-viral organisms are fungi.

20 242. The probe of claim 241 wherein said oligonucleotide comprises the sequence

CCC GAC CGT CCC TAT TAA TCA TTA CGA TGG.

243. The probe of claim 241 wherein said oligonucleotide comprise the sequence
25 CCCGACCGTCCCTATTAATCATTACGATGGTCCTAGAAAC.

244. The probe of claim 241 wherein said oligonucleotide comprises the sequence CCCGACCGTCCCTATTAATCATTACGATGG.

245. The probe of claim 241 wherein said
5 oligonucleotide comprises the sequence

CGA CTT GGC ATG AAA ACT ATT CCT TCC TGT GG.

246. The probe of claim 241 wherein said oligonucleotide comprises the sequence

GCT CTT CAT TCA ATT GTC CAC GTT CAA TTA AGC
10 AAC AAG G.

247. The probe of claim 241 wherein said oligonucleotide comprises the sequence

GCT CTG CAT TCA AAC GTC CGC GTT CAA TAA AGA
AAC AGG G.

248. A nucleotide polymer capable of
15 hybridizing to the probes of claims 242 or 243 or 244 or 245 or 246 or 247 or to the complements thereof.

249. A nucleic acid hybrid formed between an
oligonucleotide comprising a member of the group
20 consisting of oligonucleotides of the sequence

CCC GAC CGT CCC TAT TAA TCA TTA CGA TGG,

CCCGACCGTCCCTATTAATCATTACGATGGTCCTAGAAAC

CCCGACCGTCCCTATTAATCATTACGATGG

CGA CTT GGC ATG AAA ACT ATT CCT TCC TAT GG,

GCT CTT CAT TCA ATT GTC CAC GTT CAA TTA AGC
25 AAC AGG G, and

GCT CTG CAT TCA AAC GTC CGC GTT CAA TAA AGA
AAC AGG G;
and a nucleic acid sequence substantially complementary
thereto.

5 250. A nucleotide polymer comprising a
member of the group consisting of nucleotide polymers of
the structures

CCC GAC CGT CCC TAT TAA TCA TTA CGA TGG,
CCCGACCGTCCCTATTAATCATTACGATGGTCCTAGAAAC
10 CCCGACCGTCCCTATTAATCATTACGATGG
CGA CTT GGC ATG AAA ACT ATT CCT TCC TAT GG,
GCT CTT CAT TCA ATT GTC CAC GTT CAA TTA AGC
AAC AGG G, and
GCT CTG CAT TCA AAC GTC CGC GTT CAA TAA AGA
15 AAC AGG G;
and the complements thereto.

251. A nucleotide polymer capable of
hybridizing to the RNA of the phylogenetic group Fungi
in the region corresponding to position 845-880 of
20 Saccharomyces cerevisiae 18S rRNA.

252. A nucleic acid hybrid formed between a
nucleotide polymer of claim 251 and a nucleic acid
sequence substantially complementary thereto.

253. A nucleotide polymer capable of
25 hybridizing to the RNA of the phylogenetic group Fungi

in the region corresponding to position 1960-2000 of Saccharomyces cerevisiae 28S rRNA.

254. A nucleic acid hybrid formed between a nucleotide polymer of claim 253 and a nucleic acid substantially complementary thereto.

255. A nucleotide polymer capable of hybridizing to the RNA of the phylogenetic group Fungi in the region corresponding to position 1225-1270 of Saccharomyces cerevisiae 28S rRNA.

256. A nucleic acid hybrid formed between a nucleotide polymer of claim 255 and a nucleic acid substantially complementary thereto.

257. The probe of claim 31 wherein said non-viral organism is Neisseria gonorrhoeae.

258. The probe of claim 257 wherein said oligonucleotide comprises the sequence

CCG CCG CTA CCC GGT AC.

259. The probe of claim 257 wherein said oligonucleotide comprises the sequence

20 TCA TCG GCC GCC GAT ATT GGC.

260. The probe of claim 257 wherein said oligonucleotide comprises the sequence

GAG CAT TCC GCA CAT GTC AAA ACC AGG TA.

261. The probe of claim 257 wherein said oligonucleotide comprises the sequence

GAG GAT TCC GCA CAT GTC AAA ACC AGG.

262. The probe of claim 257 wherein said oligonucleotide comprises the sequence

GAG GAT TCC GCA CAT GTC AAA ACC AGG TAA.

263. The probe of claim 257 wherein said
5 oligonucleotide comprises the sequence

CCC GCT ACC CGG TAC GTTC.

264. The probe of claim 257 wherein said oligonucleotide comprises the sequence

CCG CTA CCC GGTAC GTTC.

10 265. A nucleotide polymer capable of hybridizing to the probes of claims 258 or 259 or 260 or 261 or 262 or 263 or 264 or to the complements thereof.

266. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group
15 consisting of oligonucleotides of the sequences

CCGCCGCTACCCGGTAC,

TCATCGGCCGCGGATATTGGC,

GAGCATTCCGCACATGTCAAAACCAGGTA,

GAGGATTCCGCACATGTCAAAACCAGG,

20 GAGGATTCCGCACATGTCAAAACCAGGTAA,

CCCGCTACCCGGTACGTTC, and

CCGCTACCCGGTACGTTC;

and a nucleic acid sequence substantially complementary thereto.

25 267. A nucleotide polymer comprising a

member of the group consisting of nucleotide polymers of the structures

CCGCCGCTACCCGGTAC,
TCATCGGCCGCGGATATTGGC,
5 GAGCATTCCGCACATGTCAAAACCAGGTA,
GAGGATTCCGCACATGTCAAAACCAGG,
GAGGATTCCGCACATGTCAAAACCAGGTAA,
CCCGCTACCCGGTACGTTC, and
CCGCTACCCGGTACGTTC;

10 and the complements thereto.

268. A nucleotide polymer capable of hybridizing to the RNA of the species Neisseria gonorrhoeae in the region corresponding to bases 125-150 of E. coli 16s rRNA.

15 269. A nucleic acid hybrid formed between a nucleotide polymer of claim 268 and a nucleic acid sequence substantially complementary thereto.

270. A nucleotide polymer capable of hybridizing to the RNA of the species Neisseria gonorrhoeae in the region corresponding to bases 455-
20 485 of E. coli 16s rRNA.

271. A nucleic acid hybrid formed between a nucleotide polymer of claim 270 and a nucleic acid sequence substantially complementary thereto.

25 272. A nucleotide polymer capable of hybridizing to the RNA of the species Neisseria

gonorrhoeae in the region corresponding to bases 980-1015 of E. coli 16S rRNA.

273. A nucleic acid hybrid formed between a nucleotide polymer of claim 272 and a nucleic acid sequence substantially complementary thereto.

274. The probe of claim 31 wherein said oligonucleotide is perfectly complementary to said region of rRNA.

275. The probe of claim 31 wherein said oligonucleotide is about 20 nucleotides to about 50 nucleotides in length.

276. The probe of claim 31 wherein said oligonucleotide is at least about 95% complementary to a region of rRNA.

277. A hybridization assay comprising (1) reacting together any rRNA from a sample to be assayed for a non-viral organism or organisms and an oligonucleotide probe of at least about 10 nucleotides in length which is at least about 75% complementary to a variable region of rRNA selected to be unique to said non-viral organism or organisms, (2) under conditions such that hybridization between the oligonucleotide probe and any sufficiently complementary sample rRNA can occur, and (3) observing and/or measuring said hybridization.

278. The assay of claim 277 wherein said hybridization between the oligonucleotide probe and any target sample rRNA is from at least about 10% to about 100%.

5 279. The assay of claim 277 wherein said oligonucleotide probe is cDNA.

280. The assay of claim 277 wherein said conditions include a temperature from about 25°C below T_m to about 1°C below T_m .

10 281. The assay of claim 277 which further comprises the parallel assay of a positive homologous control, or a positive heterologous control, or both.

282. The assay of claim 277 which further comprises the parallel assay of a negative control.

15 283. The assay of claim 277 wherein said conditions include agents for increased rates of hybridization.

284. The assay of claim 277 wherein said conditions are such as to promote maximum hybridization
20 between the oligonucleotide probe and any complementary sample rRNA and minimum cross-reactivity between the oligonucleotide probe and any non-complementary sample rRNA.

285. The assay of claim 277 wherein said
25 oligonucleotide probe is labelled.

286. The assay of claim 285 wherein said oligonucleotide probe is labelled with an isotopic, non-isotopic or chemiluminescent label.

287. The assay of claim 277 which further
5 comprises the release of rRNA from the cells of said non-viral organism or organisms prior to the reacting together step.

288. The assay of claim 277 wherein said non-viral organism or organisms are Mycobacterium avium,
10 Mycobacterium intracellulare, the Mycobacterium tuberculosis-complex bacteria, Mycobacterium genus, Mycoplasma pneumoniae, Legionella, Salmonella, Chlamydia trachomatis, Campylobacter, Proteus mirabilis, Enterococcus, Enterobacter cloacae, E. coli,
15 Pseudomonas group I, bacteria or fungi.

289. The assay of claim 277 wherein said labelled oligonucleotide probe is about 20 nucleotides to about 50 nucleotides in length.

290. The assay of claim 277 wherein said
20 labelled oligonucleotide probe is at least about 95% complementary to said variable region of rRNA.

291. The assay of claim 277 further
comprising the use of one or more additional
oligonucleotide probes of at least about 10 nucleotides
25 in length and which are at least about 75% complementary

to one or more additional variable regions of rRNA selected to be unique to said non-viral organisms.

292. The assay of claim 277 further comprising the use of one or more additional probes
5 which identify one or more additional non-viral organisms, thereby expanding the group of non-viral organisms to be assayed.

293. A method for preparing a probe or combination of probes for use in a qualitative or
10 quantitative hybridization assay which comprises constructing a nucleotide polymer that is sufficiently complementary to hybridize a region of DNA or rRNA selected to distinguish a target non-viral organism or group of non-viral organisms sought to be detected from
15 at least one nontarget organism or group or nontarget organisms which may be present in a sample, said region of DNA or rRNA being selected by:

comparing one or more DNA or rRNA sequences of said non-viral organism or group of non-viral organisms
20 sought to be detected with one or more DNA or rRNA sequences of said nontarget organisms or group of nontarget organisms;

aligning said DNA or rRNA sequences of said non-viral organism or group of non-viral organisms to
25 homologies with said DNA or rRNA sequences of said

nontarget organisms or group of organisms so as to identify regions of homology;

selecting said nucleotide polymer by substantially maximizing the homology of said probe
5 oligonucleotide to the regions of said DNA or rRNA of said non-viral organism or non-viral group of organisms sought to be detected while substantially minimizing the homology of said nucleotide polymer to DNA or rRNA sequences of said nontarget organisms or group of
10 organisms sought to be distinguished therefrom.

294. A method as in claim 293 wherein said nontarget organisms or group of organisms are close phylogenetic relatives of said target organisms or group of organisms.

15 295. The method of claim 292 wherein said nucleotide polymer is at least about 90% homologous to the regions of said DNA or rRNA of said non-viral organism or non-viral group of organisms sought to be detected.

20 296. The method of claim 292 wherein said probe oligonucleotide is less than about 90% homologous to DNA or rRNA sequences of said closest phylogenetic relatives sought to be distinguished therefrom.

25 297. The method of claim 293 or 294 or 295 or 296 comprising the further step of verifying said probe non-cross reactivity by hybridizing said probe

oligonucleotide to non-viral organisms or groups of non-viral organisms sought to be distinguished by said probe.

298. A method for preparing a probe for use
5 in a qualitative or quantitative hybridization assay which comprises constructing an oligonucleotide that is sufficiently complementary to hybridize a region of DNA or rRNA selected to be unique to a non-viral organism or group of non-viral organisms sought to be detected, said
10 region of DNA or rRNA being selected by:

comparing one or more DNA or rRNA sequences of said non-viral organism or group of non-viral organisms sought to be detected with one or more DNA or rRNA sequences of its closest phylogenetic relatives;

15 aligning said DNA or rRNA sequences of said non-viral organism or group of non-viral organisms to homologies with said DNA or rRNA sequences of said closest phylogenetic relatives, so as to reveal the interspecies hypervariable DNA or rRNA regions;

20 selecting said probe oligonucleotide in said interspecies hypervariable region by substantially maximizing the homology of said probe oligonucleotide to the regions of said DNA or rRNA of said non-viral organism or non-viral group of organisms sought to be
25 detected while substantially minimizing the homology of said probe oligonucleotide to DNA or rRNA sequences of

said closest phylogenetic relatives sought to be distinguished therefrom.

299. The method of claim 298 wherein said probe oligonucleotide is at least about 90% homologous
5 to the regions of said DNA or rRNA of said non-viral organism or non-viral group of organisms sought to be detected.

300. The method of claim 298 wherein said probe oligonucleotide is less than about 90% homologous
10 to DNA or rRNA sequences of said closest phylogenetic relatives sought to be distinguished therefrom.

301. The method of claim 298 or 299 or 300 comprising the further step of verifying said probe non-cross reactivity by hybridizing said probe
15 oligonucleotide to non-viral organisms or groups of non-viral organisms sought to be distinguished by said probe.

302. A probe consisting of a nucleotide polymer which is capable of hybridizing to 16S like rRNA
20 of a nonviral organism or group of organisms in the region corresponding to bases 60-100 of E.Coli 16S rRNA.

303. A nucleic acid hybrid formed between a nucleotide polymer of claim 302 and a nucleic acid sequence substantially complementary thereto.

25 304. A probe consisting of a nucleotide polymer which is capable of hybridizing to 16S like rRNA

of a nonviral organism or group of organisms in the region corresponding to bases 120-150 of E.Coli 16S rRNA.

305. A nucleic acid hybrid formed between a nucleotide polymer of claim 304 and a nucleic acid sequence substantially complementary thereto.

306. A probe consisting of a nucleotide polymer which is capable of hybridizing to 16S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 170-230 of E.Coli 16S rRNA.

307. A nucleic acid hybrid formed between a nucleotide polymer of claim 306 and a nucleic acid sequence substantially complementary thereto.

308. A probe consisting of a nucleotide polymer which is capable of hybridizing to 16S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 405-480 of E.Coli 16S rRNA.

309. A nucleic acid hybrid formed between a nucleotide polymer of claim 308 and a nucleic acid sequence substantially complementary thereto.

310. A probe consisting of a nucleotide polymer which is capable of hybridizing to 16S like rRNA of a nonviral organism or group of organisms in the

region corresponding to bases 600-670 of E.Coli 16S rRNA.

311. A nucleic acid hybrid formed between a nucleotide polymer of claim 310 and a nucleic acid sequence substantially complementary thereto.

312. A probe consisting of a nucleotide polymer which is capable of hybridizing to 16S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 820-860 of E.Coli 16S rRNA.

313. A nucleic acid hybrid formed between a nucleotide polymer of claim 312 and a nucleic acid sequence substantially complementary thereto.

314. A probe consisting of a nucleotide polymer which is capable of hybridizing to 16S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 980-1050 of E.Coli 16S rRNA.

315. A nucleic acid hybrid formed between a nucleotide polymer of claim 314 and a nucleic acid sequence substantially complementary thereto.

316. A probe consisting of a nucleotide polymer which is capable of hybridizing to 16S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 1250-1290 of E.Coli 16S rRNA.

317. A nucleic acid hybrid formed between a nucleotide polymer of claim 316 and a nucleic acid sequence substantially complementary thereto.

318. A probe consisting of a nucleotide
5 polymer which is capable of hybridizing to 23S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 270-390 of E.Coli 23S rRNA.

319. A nucleic acid hybrid formed between a
10 nucleotide polymer of claim 318 and a nucleic acid sequence substantially complementary thereto.

320. A probe consisting of a nucleotide
polymer which is capable of hybridizing to 23S like rRNA of a nonviral organism or group of organisms in the
15 region corresponding to bases 535-560 of E.Coli 23S rRNA.

321. A nucleic acid hybrid formed between a nucleotide polymer of claim 320 and a nucleic acid sequence substantially complementary thereto.

20 322. A probe consisting of a nucleotide polymer which is capable of hybridizing to 23S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 1150-1200 of E.Coli 23S rRNA.

25 323. A nucleic acid hybrid formed between a

nucleotide polymer of claim 322 and a nucleic acid sequence substantially complementary thereto.

324. A probe consisting of a nucleotide polymer which is capable of hybridizing to 23S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 1440-1600 of E.Coli 23S rRNA.

325. A nucleic acid hybrid formed between a nucleotide polymer of claim 324 and a nucleic acid sequence substantially complementary thereto.

326. A probe consisting of a nucleotide polymer which is capable of hybridizing to 23S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 1710-1750 of E.Coli 23S rRNA.

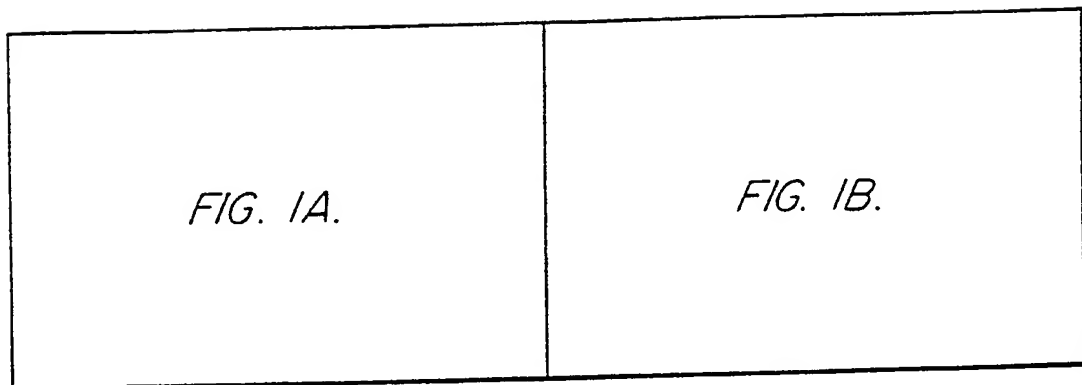
327. A nucleic acid hybrid formed between a nucleotide polymer of claim 326 and a nucleic acid sequence substantially complementary thereto.

328. A probe consisting of a nucleotide polymer which is capable of hybridizing to 23S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 2190-2330 of E.Coli 23S rRNA.

329. A nucleic acid hybrid formed between a nucleotide polymer of claim 328 and a nucleic acid sequence substantially complementary thereto.

1/28

FIG. 1.



SUBSTITUTE SHEET

FIG. 1A.

E.coli AAUUGAAGUUUGAUCAUGGCUCAGAUUGAACGCUGGCGCAGGCCUACACAUGCAAGU
E.coli AGUGGCGGACGGGUGAGUAAUGUCUGGGAAACUGCCUGAUGGAGGGGAUAAUCUACUGGAAA
E.coli GGGACCUUCGGGCCUCUUGCCAUCGGAUGUGCCAGAUUGGGAUUAAGCUAGUAGGUGGGGUA
E.coli GGAUGACCAGCCACACUGGAACUGAGACACGGUCCAGACUCCUACGGGAGGCAGUGGGG
E.coli CGCGUGUAUGAAGGCCUUCGGGUUGUAAGUACUUUCAGCGGGGAGGAAGGAGUAAAG
E.coli CACCGGCUAACUCCGUGCCAGCAGCCGCGUAUACGGAGGGUGCAAGCGUUAUCGGAUU
E.coli GAUGUGAAAUCCCCGGGCUCAACCUUGGGAACUGCAUCUGAUACUGGCAAGCUUGAGUCUCGU
E.coli UAGAGAUUCUGGAGGAUAACCGUGGCGAAGCGGCCCCUGGACGAAGACUGACGCUCAGGU
E.coli UAGUCCACGCCGUAAACGAUGUCGACUUGGAGGUUGUCCUUGAGGCGGCUUCCGGAGC
E.coli AGGUAAACUCAAUUGAAUUGACGGGGCCCGCACAAAGCGGUGGAGCAUGUGGUUUAUUC
E.coli CGGAAGUUUUCAGAGUAGAGAAUGUGCCUUCGGGAACCGUGAGACAGGUGCUGCAUGGCUGU
E.coli AACGAGCGCAACCCUUAUCCUUUUGUCCAGCGGUCCGGCCGGGAACUCAAGGAGACUGCC
E.coli AUCAUGGCCCUUACGACCGGGCUACACACGUGCUACA AUGGCGCAUACAAGAGAGCGAC
E.coli UCCGGAUUGGAGUCUGCAACUCGACUCCAUAGAUGCGGAUCCGUAGUAAUCCGUGGAUCAGA
E.coli GCCCGUCACCAUGGGAGUGGGUUGCAAAAGAGUAGGUAGCUUAAACCUUGGGAGGGCGC
E.coli CAAGGUAAACCGUAGGGGAACCUUGCGGUUGGAUACCUCCUUA

2/28

SUBSTITUTE SHEET

3/28

FIG. 1B.

CGAACGGUAAACAGGAAGAGCUUGCUUUCUUUGCUGACG 100
CGGUAGCUAAUACCGCAUAAACGUGCGAAGACCAAGAG 200
ACGGCUCACCUAGGGGACGAUCCCUAGCUGGUCUGAGA 300
AAUAUUGCACAAUGGGCGCAAGCCUGAUGCAGCCAUGC 400
UUAUAACCUUUGCUCAUUGACGUUACCCGCAGAGAAG 500
ACUGGGCGUAAAGCGCACGCAGCGGCUUUGUUAAGUCA 600
AGAGGGGGUAGAAUCCAGGUGUAGCGGUGAAAUGCG 700
GCGAAAGCGUGGGGAGCAAACAGGAUUAAGAUACCCUGG 800
UAACGCGUUAAGUCGACCGGCCUGGGGAGUACGGCCGCA 900
GAUGCAACGGGAAGAACCUUACCUUGGUCUUGACAUCCA 1000
CGUCAGCUCGUGUUGUGAAAUGUUGGGUUAAGUCCCGC 1100
AGUGAUAAACUGGAGGAAGGUGGGGAUGACGUCAAGUC 1200
CUCGGAGAGCAAGCGGACCUCUAUAAAGUGCGUGCUAG 1300
AUGCCACGGUGAAUACGUUCCCGGGCCUUGUACACACC 1400
UUACCACUUUGAUUCAUGACUGGGGUGAAGUCGUAA 1500
1542

SUBSTITUTE SHEET

CGK00033318

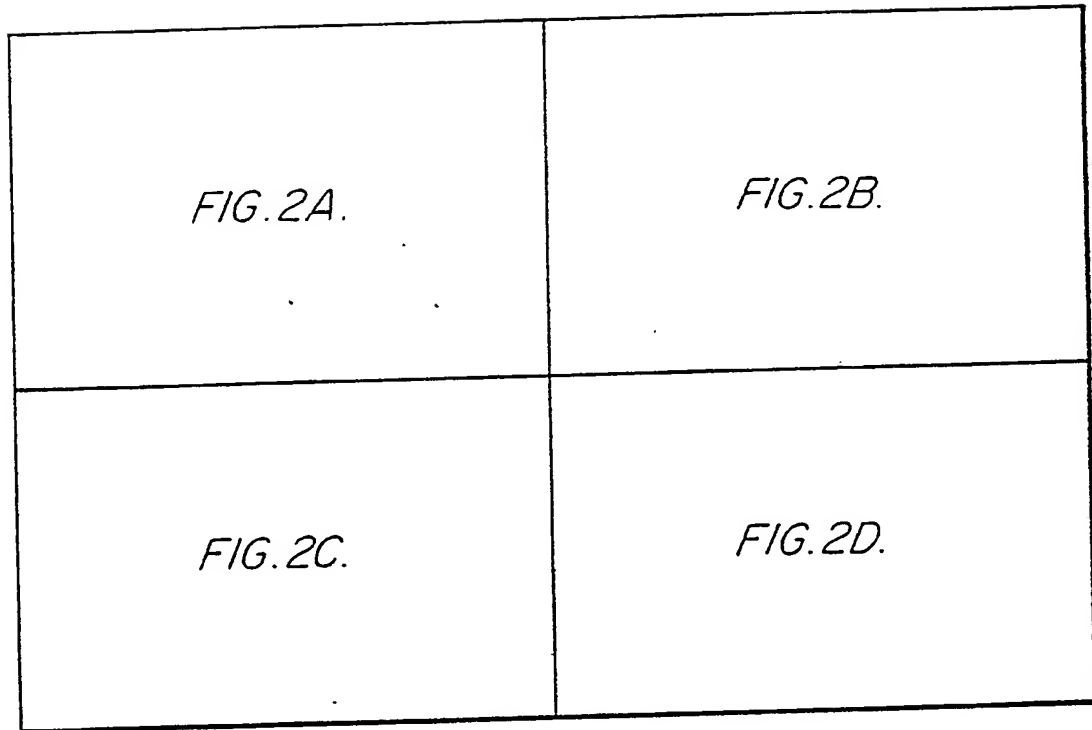
FIG. 2.

FIG. 2A.

E.coli GGUAAGCGACUAAGCGUACACGGUGGAUGCCUGGCAGUCAGAGGCGAUGAAGGACGUGCU
E.coli AUAACCGGCGAUUCCGAUUGGGAAACCCAGUGUGUUUCGACACACUAUCAUUAACUGAAU
E.coli CUAAGUACCCCGAGGAAAGAAAUCAACCGAGAUUCCCCCAGUAGCGGCGAGCGAACGGGGA
E.coli GCGUCUGGAAAGCGCGCGGAUACAGGGUGACAGCCCCCGUACACAAAUAGCAUUCUGUGA
E.coli AAUAGGGGGACCAUCCUCCAAGGCUAAAUACUCCUGACUGACCGAUAGUGAACCCAGUACC
E.coli AAAAAGAACCCUGAAACCGUGUACGUACAGCAGUGGGAGCACGCUUAGGCGUGUGACUGCGU
E.coli CAAGGUUAACCGAAUAGGGGAGCCGAAGGAAACCGAGUCUUAACUGGGCGUUAAAGUUGCAG
E.coli GUUGAAGGUUGGUAAACACUAAACUGGAGGACCGAACCGACUAUUGUUGAAAUUAGCGGAU
E.coli GAUAGCUGGUUUCUCCCGAAAGCUAUUAGGUAGCGCCUCCUGAAUUAUCUCCGGGGUAG
E.coli CCCGAUGCAAACUGCGAAUACCGGAGAAUGUUUAUCACGGGAGACACACGGCGGUGCUAACG
E.coli AGGUCCCAAAGUCAUGGUUAAAGUGGGAAACGAUGUGGGAAGGCCACAGACGCCAGGAUGUUG
E.coli UCACUGGUCGAGUCGGGCCUGCGCGGAAGAUUAACGGGGCUAAACCAUGCACCGAAGCUGCG
E.coli UGUAAAGCCUGCGAAGGUGUGUGAGGCAUGCUGGAGGUUAUCAGAGUGCGGAUUGCUGACA
E.coli AAGACCAAGGUUCCUGUCCAACGUUAAUCGGGGCAGGGUGAGUCGACCCCUAAGCGGAGGC

5/28

SUBSTITUTE SHEET

6/28

FIG. 2B.

AAUCUGCGAUAGCGUGCGGUAAGGUGAUAGAACCGUU 100
CCAUAGGUUAAUGAGGGCGAACCGGGGGAACUGAAACAU 200
GCAGCCCAGAGCCUGAAUCAGUGUGUGUUAAGUGGAA 300
GCUCGAUGAGUAGGGCGGGAACGUGGUAUCCUGUCUG 400
GUGAGGGAAGGCGAAAGAAACCCCGGCGAGGGGAGUG 500
ACCUUUUGUAUAUGGGUCAGCGACUUAUAUUCUGUAG 600
GGUAUAGACCCGAAACCCGGUGAUCUAGCCAUGGGCAG 700
GACUUGUGGCGUGGGGUGAAAGGCCAAUCAAACCGGGA 800
AGCACUGUUUCGGCAAGGGGUGUAUCCCGACUUAACCAA 900
UCCGUCGUGAAGAGGGAACAACCCAGACCGCCAGCUA 1000
GCUUAGAAGCAGCCAUCAUUUAAAGAAAGCGUAUAGC 1100
GCAGCGACGCUUAUGCGGUUGUUGGUAGGGGAGCGUUC 1200
UAAGUAACGAUAAAGCGGGUGGAAAGCCCGCUCGCCGG 1300
CGAAAGGCGUAGUCGAUGGGGAAACAGGUUAUAUUCU 1400

SUBMITTAL COPY

FIG. 2C.

E.coli GUACUUGGUGUUAUCUGCGAAGGGGGACGGAGAAGGCUAUGUUGCGCGGACGGUUGUCC
 E.coli GAAAUCAAGGCUGAGGCGUGAUGACGAGGCACUACGGUGCUGAAGCAACAAGCCCCUGCU
 E.coli GUACCCCAACCGACACAGGUGGUCAGGUAGAGAAUACCAAGGCCUUGAGAGAACUCGGGU
 E.coli AGGCACGUGAUAGUAGGUGAGGUCCUCGCGGAUGGAGCUGAAUACAGUCGAAGAUACCA
 E.coli AAACACGAAAGUGGACGUAUACGGUGUGACGCCUGCCCCGUGCCGGAAGGUUAUUGAUGGG
 E.coli ACGGGGCCGUAACUAUAACGGUCCUAAGGUAGCGAAUUCUUGUCGGGUAAGUUCGGACC
 E.coli CGAGACUCAGUGAAUUGAACUCGCGUGUGAAGAUAGCAGUGUACCCGCGCAAGACGGAAAGA
 E.coli AGCCUUGAUGUGAUGAUAGGUGGGAGGCCUUUGAAGUGUGGACGCCAGUCUGCAUGGAGCCG
 E.coli GUUGACCCGUAUACCGGUUGCGGACAGUGUCUGGUGGGUAGUUUGACUGGGCGGUCUCU
 E.coli CUGGUCGGACAUCAGGAGGUUAGUGCAUAGGCAUAAAGCAUCCGCGGUAACAGGCUGAUC
 E.coli UUCUGAAUGGAAGGCCAUCGCUCAACGGAUAAAGGUACUCCGGGUAUACAGGCUGAUAC
 E.coli CGAUGUCGGCUCAUCAUCCUGGGGUGAAGUAGGUCCCAAGGUUAGGCUUGGCCCAU
 E.coli CAGUUCGGUCCUAUCUGCCGUGGGCGUGGAGAACUGAGGGGGGCUCCUAGUACGAGA
 E.coli UGCCAAUGGCACUGCCCCGUAGCUAAAUAGCGGAAGAGAUAGUGCUGAAAGCAUCUAAGCAC
 E.coli GGGUCCUGAAGGAACGUUGAAGACGACGACGUUGAUAGGCCGGGUGUGUAAGCGCAGCGAUG
 E.coli CCUU

7/28

SUBSTITUTE SHEET

8/28

FIG. 2D.

CGGUUUAAGCGUGUAGGCUGGUUUUCCAGGCAAUCCG 1500
UCCAGGAAAGCCUCUAAGCAUCAGGUACAUAUAAUC 1600
GAAGAACUAGGCAAAUUGGUGCCGUAAUUCGGGAGA 1700
GCUGGCUGCAACUGUUUAUUAAAAACACAGCACUGUGC 1800
GUUAGCGCAAGCGAAGCUCUUGAUCAAGCCCGGUAA 1900
UGCACGAUGGCGUAAUGAUGGCCAGGCUGUCUCCACC 2000
CCCCGUGAACCUUUAUAUAGCUUGACACUGAACAUUG 2100
ACCUUGAAUACCAACCCUUUAAUGUUUGAUUUAUUAAC 2200
CCUAAAGAGUAAACGGAGGAGCACGAAGGUUGGCUAUC 2300
GCGAGCAGGUGCGAAAGCAGGUCUAUAGUACCGGUGG 2400
CGCCCAAGAGUUCAUAUCGACGGCGGUGUUUGGCACCU 2500
UAAAGUGUACGCGAGCUGGGUUUAGAACGUCGUGAGA 2600
GGACCGGAGUGGACGCAUCACUGGUGUUCGGGUUGUCA 2700
GAAACUUGCCCCGAGAUGAGUUUCCUGACCCUUUAA 2800
CGUUGAGCUAAACCGGUACUAUGAACCGUGAGGCUUAA 2900
2904

SUBSTITUTE SHEET

CGK00033323

9/28

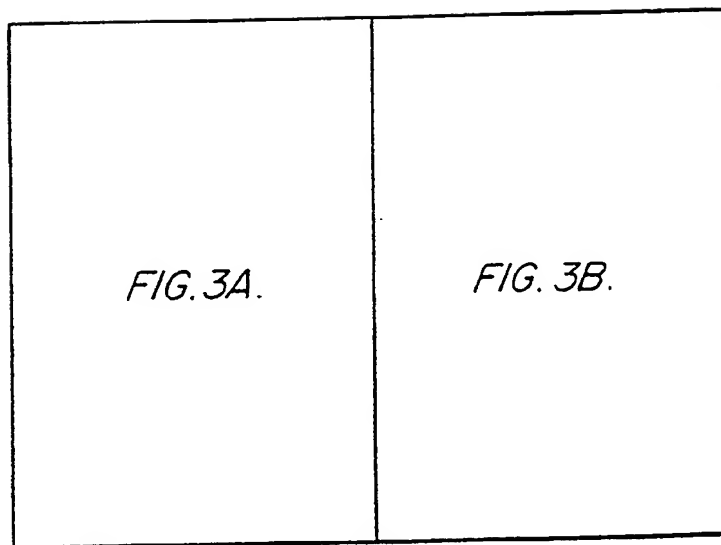


FIG. 3.

10/28

FIG. 3A.

E.coli UGCCUGGCGGCCGUAGCGCGGUGGUCCCACCUGACCCCAUGCCGAACUC

E.coli AGUAGGGAACUGCCAGGCAU

11/28

FIG. 3B.

AGAAGUGAAACGCCGUAGCGCCGAUGGUAGUGUGGGGUCUCCCCAUGCGAG 100

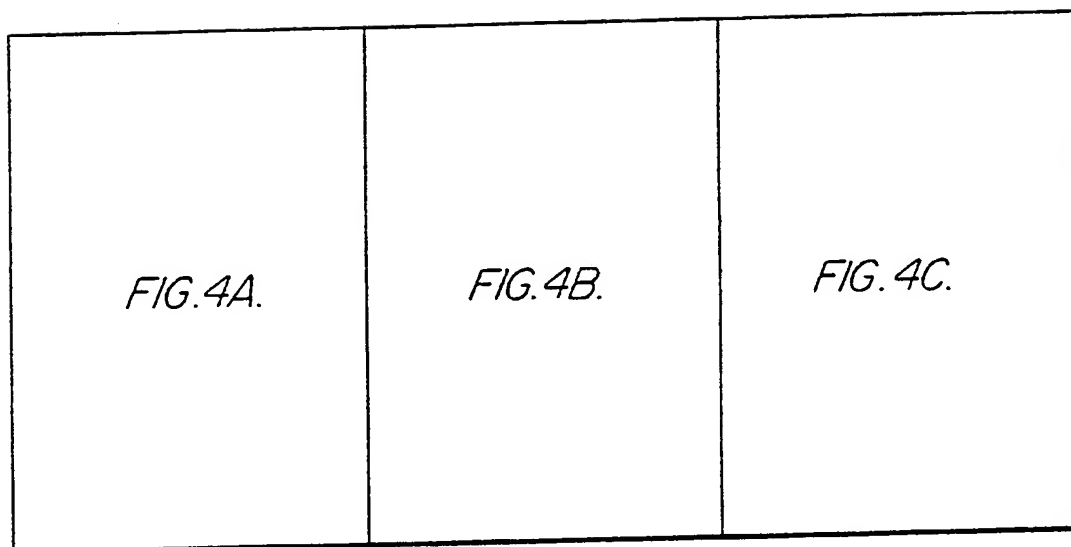
120

SUBSTITUTE SHEET

CGK00033326

12/28

FIG. 4.



SUBSTITUTE SHEET

13/28

S.cerevisiae.sc UAUCUGGUUGAUCCUGCCAGUAGUCAUAUGCUUGUCUCAA
S.cerevisiae.sc UUAAAUCAGUUAUCGUUUAUUUGAUAGUCCUUUACUACAU
S.cerevisiae.sc GAUGUAUUUAUUAGAUAAAAAUCAAUGUCUUCGCACUCUU
S.cerevisiae.sc AUUUCUGCCCUAUCAACUUUCGAUGGUAGGAUAGUGGCCUA
S.cerevisiae.sc ACGGCUACCACAUCCAAGGAAGGCAGCAGGCGCGCAAUUA
S.cerevisiae.sc UUGUAAUUGGAAUGAGUACAAUGUAAAUACCUUAACGAGGA
S.cerevisiae.sc AUUAAAGUUGUUGCAGUUA AAAAGCUCGUAGUUGAACUUUG
S.cerevisiae.sc UGGCUAACCUUGAGUCCUUGUGGCUCUUGGCGAACCAAGGAC
S.cerevisiae.sc GGAAUAAUAGAAUAGGACGUUUGGUUCUAUUUUGUUGGUUU
S.cerevisiae.sc GUGAAAUUCUUGGAUUUAUUGAAGACUAACUACUGCGAAAG
S.cerevisiae.sc GAUACCGUCGUAGUCUUAACCAUAAACUAUGCCGACUAGGG
S.cerevisiae.sc GUUCUGGGGGGAGUAUGGUCGCAAAGGCUGAAACUUAAGG
S.cerevisiae.sc GAAACUCACCAGGUCCAGACACAAUAAGGAUUGACAGAUUG
S.cerevisiae.sc AUUUGUCUGCUUAAUUGCGAUACGAACGAGACCUUAACCU
S.cerevisiae.sc AAGCCGAUGGAAGUUUGAGGCAUAACAGGUCUGUGAUGCC
S.cerevisiae.sc GCCGAGAGGUCUUGGUAAUCUUGUGAAACUCCGUCGUGCUG
S.cerevisiae.sc CAGCUUGCGUUGAUUACGUCCCUGCCCUUUGUACACACCGC
S.cerevisiae.sc GCAACUCCAUCUCAGAGCGGAGAAUUUGGACAAACUUGGUC
S.cerevisiae.sc UA

FIG. 4A.

SUBSTITUTE SHEET

14/28

GAUUAAGCCAUGCAUGUCUAAGUAUAAGCAAUUUAUACAGUGAAACUGCGAA
GGUAUAACCGUGGUAAUUCUAGAGCUAAUACAUGCUUAAAAUCUCGACCCUU
UGAUGAUUCAUAAUAACUUUUCGAAUCGCAUGGCCUUGUGCUGGCGAUGGUU
CCAUGGUUUAACGGGUAAACGGGGAAUAAGGGUUCGAUUCCGGAGAGGGAGC
CCCAAUCCUAAUUCAGGGAGGUAGUGACAAUAAUAACGAUACAGGGCCCAU
ACAAUUGGAGGGCAAGUCUGGUGCCAGCAGCCGCGGUAAUUCAGCUCCA
GGCCCGGUUGGCCGGUCCGAUUUUUUCGUGUACUGGAUUUCCAACGGGGCCU
UUUUACUUUGAAAAAAUUAAGAGUGUCAAAGCAGGCGUAUUGCUCGAAUUA
CUAGGACCAUCGUAAUGAUUAAUAGGGACGGUCGGGGGCAUCGGUAUUCAAU
CGUUUGCCAAGGACGUUUUCGUUAAUCAAGAACGAAAGUUGAGGGAUUCGAAG
AUCGGGUGGUGUUUUUUUAAUGACCCACUCGGUACCUUACGAGAAUCAAG
AAUUGACGGAAGGGCACCACCAGGAGUGGAGCCUGCGGCUUAAUUUGACUCA
AGAGCUCUUUCUUGAUUUUGUGGGUGGUGGUGCAUGGCCGUUUCUCAGUUGG
ACUAAAUAGUGGUGCUAGCAUUUGCUGGUUAUCCACUUCUAGAGGGACU
CUUAGAACGUUCUGGGCCGCACGCGCGCUACACUGACGGAGCCAGCGAGUCU
GGGAUAGAGCAUUGUAAUUAUUGCUCUUAACGAGGAAUUCUAGUAAGCGC
CCGUCGCUAGUACCGAUUGAAUGGCUUAGUGAGGCCUCAGGAUCUGCUUAGA
AUUUGGAGGAACUAAAAGUCGUAACAAGGUUCCGUAGGUGAACCGCGGAA

FIG. 4B.

SUBSTITUTE SHEET

UGGCUCA 100
UGGAAGA 200
CAUUCAA 300
CUGAGAA 400
UCGGGUC 500
AGCGUAU 600
UUCCUUC 700
UUAGCAU 800
UGUCGAG 900
ACGAUCU 1000
UCUUUGG 1100
ACACGGG 1200
UGGAGUG 1300
CGGUUUC 1400
AACCUUG 1500
AAGUCAU 1600
GAAGGGG 1700
GGAUCAU 1800
1802

FIG. 4C.

SUBSTITUTE SHEET

16/28

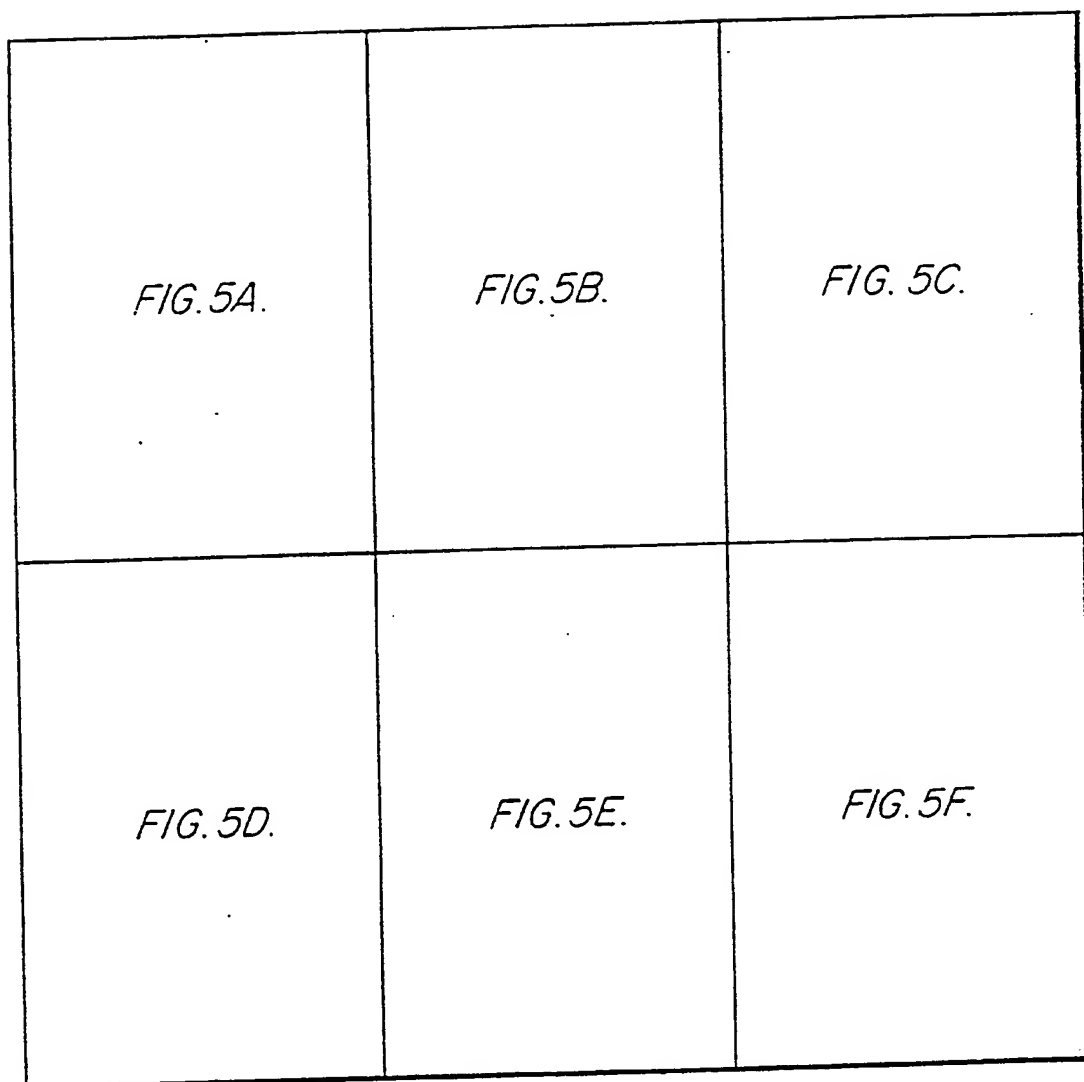
FIG. 5.

FIG. 5A.

17/28

S.cerevisiae AAACUUUCAACAACGGAUCUCUUGGUUCUCGCAUCGAUGAAGA
S.cerevisiae UUGGUUAUCCAGGGGGCAUGCCUGUUUGAGCGUCAUUUGUUUG
S.cerevisiae UAGUAACGGCGAGUGAAGCGGCAAAAGCUCAAAUUUGAAAUCU
S.cerevisiae ACGUCAUAGAGGGUGAGCAUCCCGUGUGGCGAGGAGUGCGGUU
S.cerevisiae AAUAUUGGCGAGAGACCGAUAGCGAACAAGUACAGUGAUGGAA
S.cerevisiae UGUUUUGUGCCCUCUGCUCCUUGUGGGUAGGGGAUUCUGCAU
S.cerevisiae GUGGGAAUACUGCCAGCUGGGACUGAGGACUGCGACGUAAGUC
S.cerevisiae GGGUGUAAAACCAUACGCGUAAUGAAAGUGAACGUAGGUUGG
S.cerevisiae CGAAAGAUGGUGAACUAUGCCUGAAUAGGGUGAAGCCAGAGGA
S.cerevisiae GAACCAUCUAGUAGCUGGUUCCUGCCGAAGUUUCCUCAGGAU
S.cerevisiae UCAAACUUUAAAUAUGUAAGAAGUCCUUGUUACUAAUUGAAC
S.cerevisiae GAGUUAAGGUGCCGGAUACACGCUCAUCAGACACCACAAAAG
S.cerevisiae CGA AUGAACUAGCCCUGAAAAUGGAUGGCGCUCAAGCGUGUUA
S.cerevisiae GUAAGGUCGGGUCGAACGGCCUCUAGUGCAGAUUCUUGGUGGUA
S.cerevisiae AGUCGAUCCUAAGAGAUGGGGAAGCUCCGUUCAAAGGCCUGA
S.cerevisiae UGAAUGUGGAGACGUCGGCGCGAGCCCUGGGAGGAGUUUUCU
S.cerevisiae UGCUGGCUCGGUGCGCUUGUGACGGCCCGUGAAAUCCACAG
S.cerevisiae UAAUGUAGAUAAAGGGAAGUCGGCAAAUAGAUCGGUAACUUCG
S.cerevisiae GGGGCUUGCUCUGCUAGGCGGACUACUUGCGUGCCUUGUUGUA
S.cerevisiae GAAUCUGACUGUCUAAUUAAAACAUAGCAUUGCGAUGGUCAGA
S.cerevisiae ACGGCGGGAGUAACUAUGACUCUCUUAAGGUAGCCAAAUGCCU
S.cerevisiae GCCAAGGGAACGGGCUUGGCAGAAUCAGCGGGGAAGAAGACC

SUBSTITUTE SHEET

CGK00033332

FIG. 5B.

18/28

ACGCAGCGAAAUGCGAUACGUAAUGUGAAUUGCAGAAUCCGUGAAUCAUCG
* ACCUCAAAUCAGGUAGGAGUACCCGCUGAACUUAAGCAUAUCAUAAGCGGA
9 GGUACCUUCGGUGCCCGAGUUGUAAUUUGGAGAGGGCAACUUUGGGGCCGU
CUUUGUAAAGUGCCUUCGAAGAGUCGAGUUGUUUGGGAUUGCAGCUCUAAGU
AGAUGAAAAGAACUUUGAAAAGAGAGUGAAAAAGUACGUGAAAUUGUUGAAA
UUCACUGGGCCAGCAUCAGUUUUGGUGGCAGGAUAAAUCCAUAAGGAUUGUAG
AAGGAUGCUGGCAUAAUGGUUAUAUGCCGCCCGUCUUGAAACACGGACCAAG
GGCCUCGCAAGAGGUGCACAUCGACCGAUCCUGAUGUCUUCGGAUGGAUUU
AACUCUGGUGGAGGCUCGUAGCGGUUCUGACGUGCAAUCGAUCGUCGAAUU
AGCAGAAGCUCGUUUCAGUUUAUGAGGUAAAGCGAAUGAUUAGAGGUUCCG
GUGGACAUUUGAAUGAAGAGCUUUUAGUGGGCCAUUUUUGGUAAGCAGAACU
GUGUUAGUUCAUCUAGACAGCCGGACGGUGGCCAUGGAAGUCGGAUCCGCU
CCUAUACUCUACCGUCAGGGUUGAUUAUGAUGCCCGACGAGUAGGCAGGCGU
GUAGCAAUAUUCAAAUGAGAACUUUGAAGACUGAAGUGGGGAAAGGUUCCA
UUUUAUGCAGGCCACCAUCGAAAGGGAAUCCGGUAAGAUAUCCGGAACUUGGA
UUCUUCUUAACAGCUUAUCACCCCGGAAUUGGUUUAUCCGGAGAUGGGGUCU
GAAGGAAUAGUUUUAUGCUAGGUCGUACUGAUAAACCGCAGCAGGUCUCCAA
9 GGAUAAGGAUUGGCUCUAAGGGUCGGGUAGUGAGGGCCUUGGUCAGACGCAG
GACGGCCUUGGUAGGUCUCUUGUAGACCGUCGCUUGCACAAUUAACAGAU
AAGUGAUGUUGACGCAAUGUGAUUUCUGCCCAGUGCUCUGAAUGUCAAGUG
CGUCAUCUAAUAGUGACGCGCAUGAAUGGAUUAACGAGAUUCCACUGUCC
CUGUUGAGCUUGACUCUAGUUUGACAUGUGAAGAGACAUAAGAGGGUGUAGA

SUBSTITUTE SHEET

19/28

AAUCUUUGAACGCACAUUGCGCCCC 120
GGAAAAGAAACCAACCGGAUUGCCU 240
CCUUGUCUAUGUCCUUGGAACAGG 360
GGGUGGUAAAUCCAUCUAAAGCUA 480
GGGAAGGGCAUUUGAUCAGACAUGG 600
CUUGCCUCGGUAAGUAUUAUAGCCU 720
GAGUCUAACGUCUAUGCGAGUGUUU 840
GAGUAAGAGCAUAGCUGUUGGGACC 960
UGGGUAUAGGGGCGAAAGACUAAUC 1080
GGGUCGAAAUGACCUUGACCUAUUC 1200
GGCGAUGCGGGAUGAACCGAACGUA 1320
AAGGAGUGUGUAACAACUCACCGGC 1440
GGAGGUCAGUGACGAAGCCUAGACC 1560
CGUCAACAGCAGUUGGACGUGGGUU 1680
UAUGGAUUCUUCACGGUAACGUAAC 1800
UAUGGCUGGAAGAGGCCAGCACCUU 1920
GGUGAACAGCCUCUAGUUGAUAGAA 2040
CGGGCGUGCUUGUGGACUGCUUGGU 2160
AACUUAGAACUGGUACGGACAAGGG 2280
AAGAAUUAACCAAGCGCGAGUAA 2400
CUAUCUACUAUCUAGCGAAACCACA 2520
AUAAGUGGGAGCUUCGGCGCCAGUG 2640

FIG. 5C.

SUBSTITUTE SHEET

CGK00033334

20/28

S.cerevisiae AAUACCACUACCUUUUAGUUUCUUUACUUAUCAAUGAAGC
S.cerevisiae UGGGGAGUUUGGCUGGGGCGGCACAUCUGUAAAACGAUAACGC
S.cerevisiae GUGUGAAUACAAACCAUUGAAAGUGUGGCCUAUCGAUCCUUUA
S.cerevisiae AGCGACAUUGCUIUUUGAUUCUUCGAUGUCGGCUCUCCUAUC
S.cerevisiae AGACAGGUUAGUUUUACCCUACUGAUGAAUGUUACCAGCAAUA
S.cerevisiae AAGCACCAUCCGCUGGAUUAUGGCUGAACGCCUCUAAGUCAGA
S.cerevisiae UGAACCAUAGCAGGCUAGCAACGGUGCACUUGGCGGAAAGGCC
S.cerevisiae GGUAUUGUAAGCGGUAGAGUAGCCUUGUUGUUACGAUCUGCUG
S.cerevisiae
S.cerevisiae

FIG. 5D.

21/28

GGAGCUGGAAUUCAUUUUCCACGUUCUAGCAUUCAAGGUCCCAUUCGGGGCU
AGAUGUCCUAAGGGGGGCUCAUGGAGAACAGAAAUCUCCAGUAGAACAAAAG
GUCCCUCGGAAUUUGAGGCUAGAGGUGCCAGAAAAGUUACCACAGGGGAUAC
AUACCGAAGCAGAAUUCGGUAAGCGUUGGAUUGUUCACCCACUAAUAGGGAA
GUAUUUGAACUUAGUACGAGAGGAACAGUUCAUUCGGAUAAUUGGUUUUUGC
AUCCAUGCUAGAACGCGGUGAUUUUCUUUGCUCCACACAAUUAUAGAUGGAUAC
UUGGGUGCUUGCUGGCGAAUUGCAAUGUCAUUUUGCGUGGGGAUAAAUCAUU
AGAUUAAGCCUUUGUUGUCUGAUUUGU

FIG. 5E.

SUBSTITUTE SHEET

CGK00033336

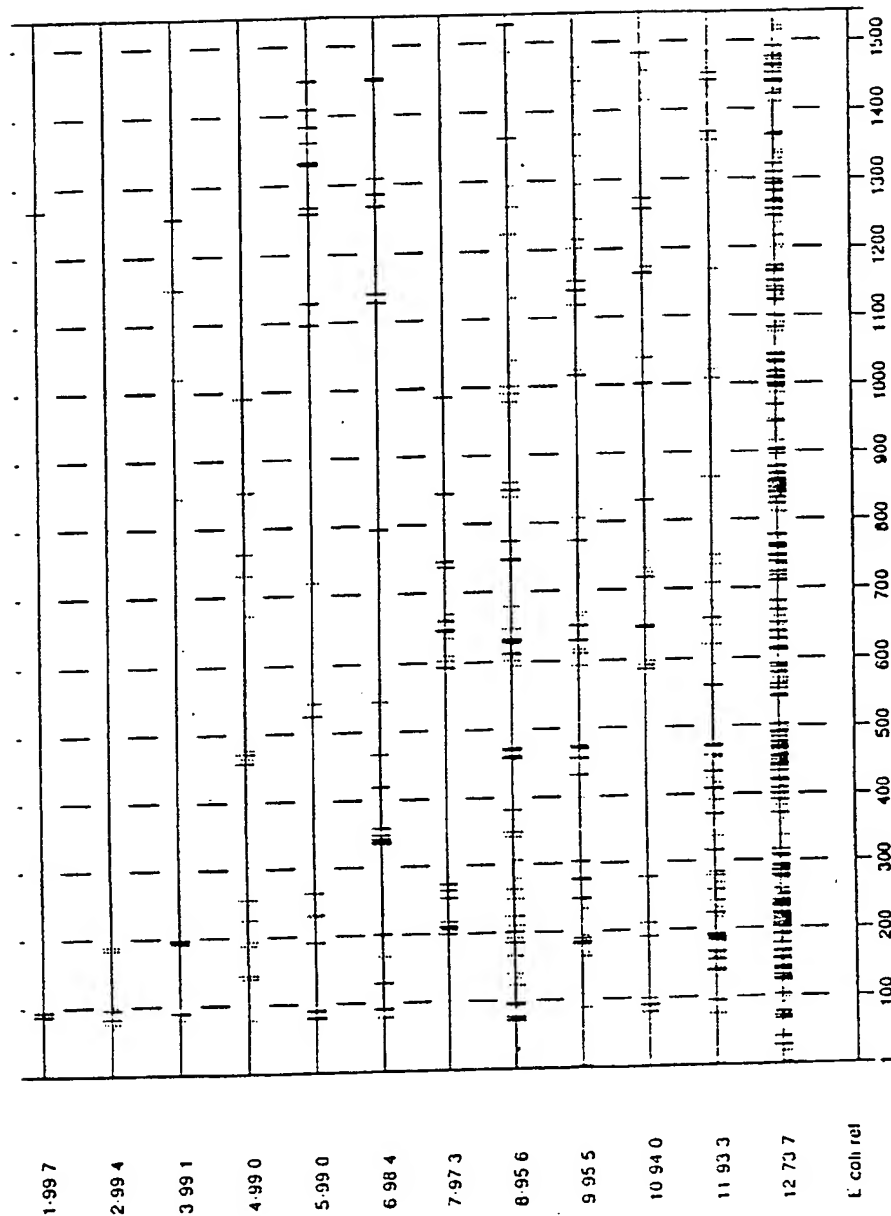
22/28

GAUCCGGGUUGAAGACAUUGUCAGG 2760
GGUAAAGCCCCUUAGUUUGAUUUCA 2880
UGGCUUGUGGCAGUCAAGCGUUCAU 3000
CAUGAGCUGGGUUUAGACCGUCGUG 3120
GGCUGUCUGAUCAGGCAUUGCCGCG 3240
GAAUAAGGCGUCCUUGUGGCGUCGC 3360
UGUAUACGACUUAGAUGUACAACGG 3480
3550
3550
3550

FIG. 5F.

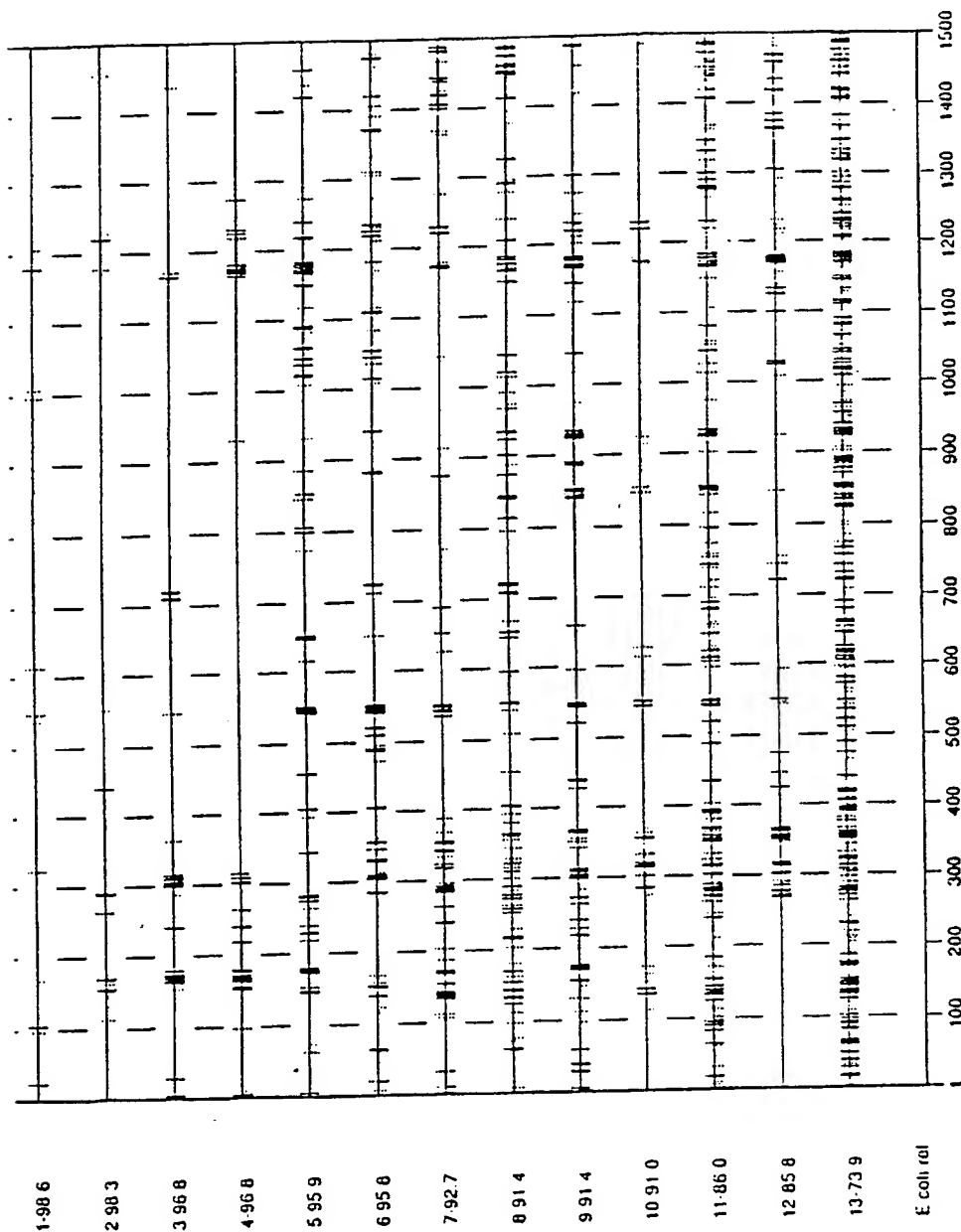
SUBSTITUTE SHEET

FIG. 6



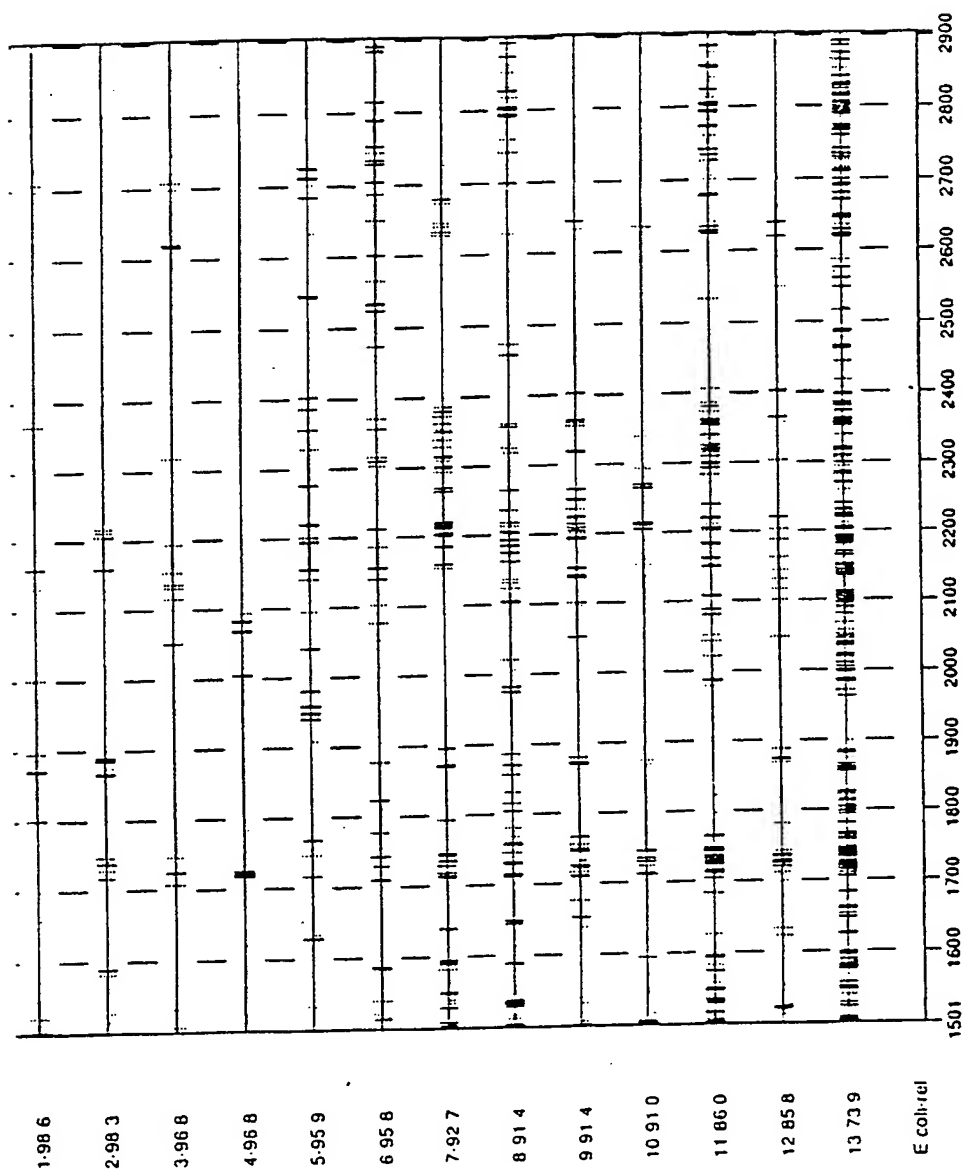
SUBSTITUTE SHEET

FIG. 7



25/28

FIG. 8



SUBSTITUTE SHEET

26/23

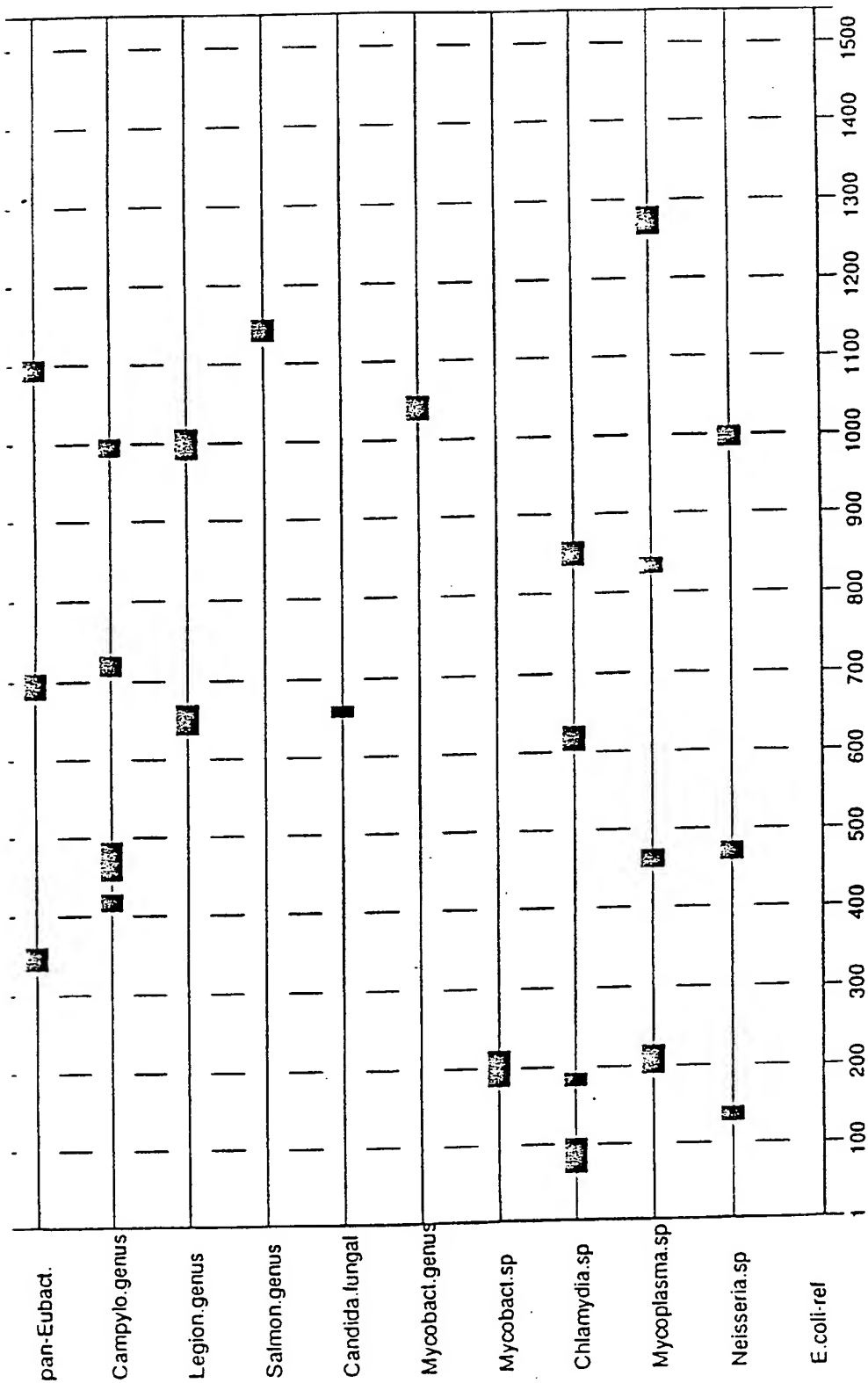


FIG.9

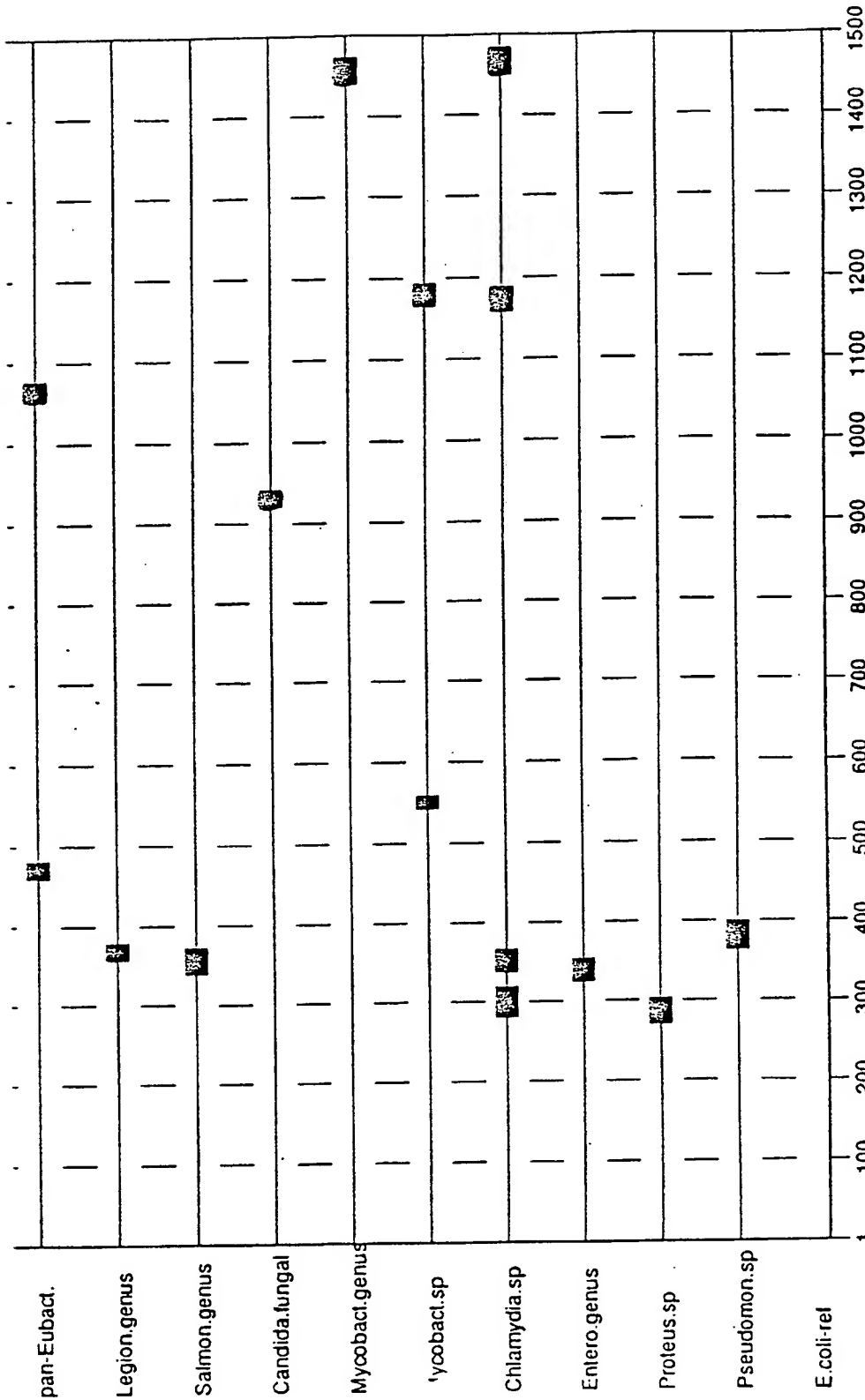


FIG.10

28/28

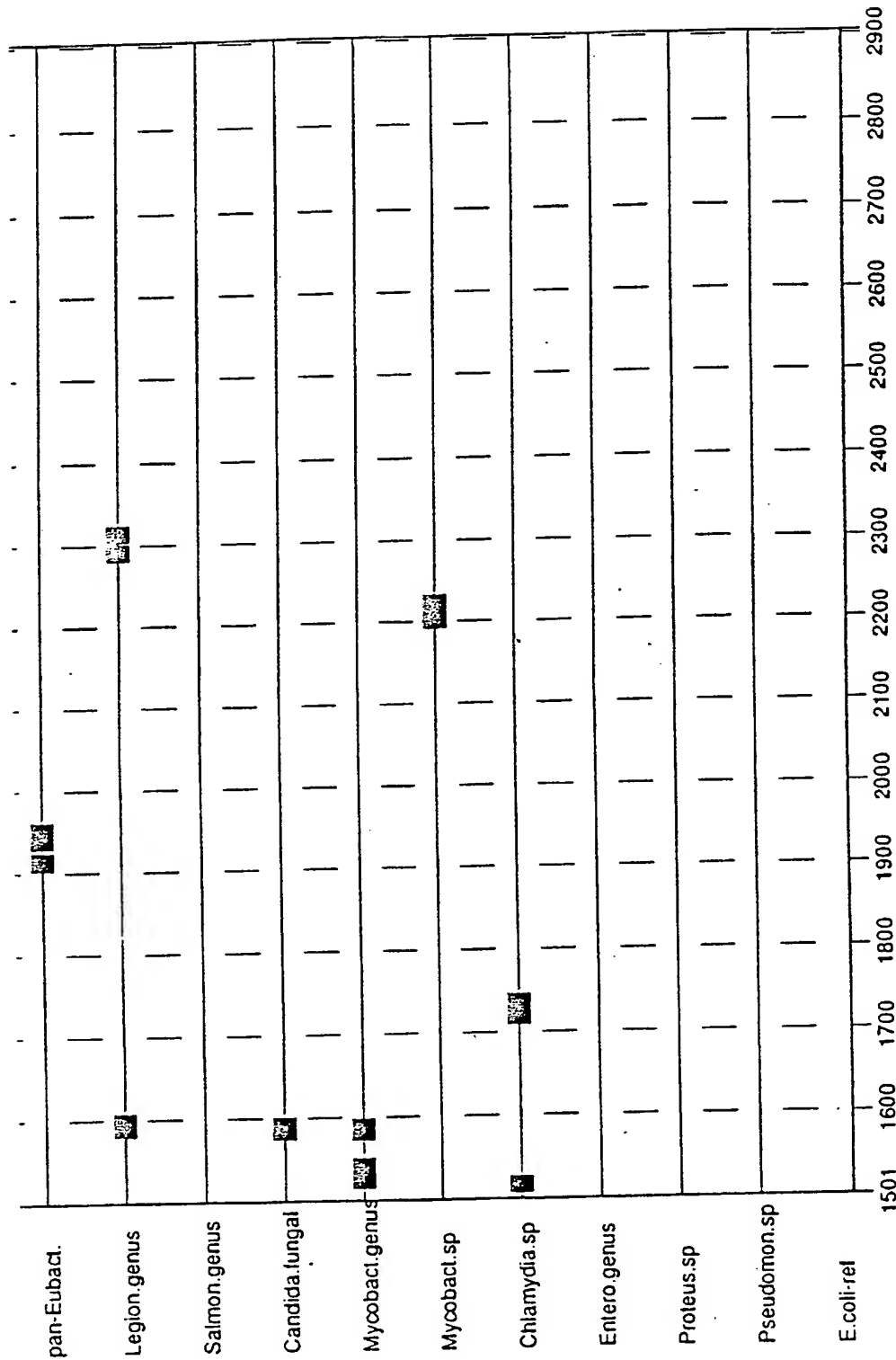


FIG. II

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US87/03009

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ¹		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (M): C12Q 1/68; C07H 21/00		
US CL: 435/6; 536/27		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/6, 34, 803, 5 436/501; 536/27; 935/78	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
COMPUTER SEARCH: DNA STAR/GENEMAN, APS, CHEMICAL ABSTRACTS, BIOSIS		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	WO, A, 84/02721 (GEN-PROBE PARTNERS) 19 July 1984. See pages 1 (lines 5-14), 3 (lines 13-22), 6 (lines 1-2), 22 (line 3-6), 27 (lines 6-28), 28 (lines 5-9), 29 (lines 10-13), 30 (lines 20-30), 33 (lines 23-30), 40 (lines 12-40), 41 (lines 1-5; 25-30), 48 (lines 3-10), 56 (lines 15-27), 57 (lines 10-18; 25-30), 58 (line 10).	1-329
Y	WO, A, 83/01073 (WEBSTER) 31 March 1983. See pages 10 (lines 5-10, 25-30), 11 (lines 8-22, 29-35), 12 (lines 3 and 14), 13 (lines 32-35), 15 (lines 1-11), 16 (lines 2-8, 14- 20, 26-29), 17 (lines 15-24), 18 (lines 15- 24), 19 (lines 8-30), 20 (lines 1-9, 24-25), 23 (lines 1-14, 30-32), 25 (lines 21-31), 26 (lines 20-32), 34 (lines 1-8, 11-17).	1-329
Y	American Clinical Products Review, issued November 1986 (Fairfield, CT) D.E. Kohne "Application of DNA probe tests to the diagnosis of infectious diseases". See pages 22, 24 and 25.	1-329
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁵ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²		Date of Mailing of this International Search Report ³
13 January 1988		16 MAR 1988
International Searching Authority ¹		Signature of Authorized Officer ¹⁰
ISA/US		Jeremy M. Jay

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹³ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁴
Y	Proceedings of the National Academy of Sciences, USA, Volume 82, issued October 1985 (Washington DC), D.J. Lane et al., "Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses". See pages 6955, 6956 and 6958.	1-329
Y	Proceedings of the National Academy of Sciences, USA, Volume 82, issued February 1985 (Washington, DC), M.J. Rogers et al "Construction of the mycoplasma evolutionary tree from 5S rRNA sequence data. See page 1160.	1-329
Y	Journal of Bacteriology, Volume 167, Number 2, issued August 1986 (Washington, DC.) W.G. Weisburg et al., "Eubacterial Origin of Chlamydiae". See pages 570 and 571.	1-329
Y	Proceedings National Academy Of Sciences, USA, Volume 77, Number 1, issued January 1980 (Washington, DC) J. Brosius et al "Complete nucleotide sequence of a 23S ribosomal RNA gene from <u>Escherichia coli</u> ". See page 201.	33,62-67, 78-83,117- 122,145- 156,185, 186,192, 193,199, 200,209- 212,235- 240,288, 318-329
Y	Proceedings National Academy Of Sciences, USA, Volume 75, Number 10, issued October 1978, (Washington, DC) J.Brosius et al, "Complete nucleotide sequences of a 16S ribosomal RNA gene from <u>Escherichia coli</u> ". See page 4801..	33,40,41, 47,48,60, 61,76,77, 93-100, 113-116, 137-144, 165-172, 178,179, 207,208, 211-212, 216,217, 229-234, 268-272, 288,302- 317